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The purpose of this research was to characterize the factors controlling the expression of c-myc and other genes that are commonly mis-regulated in transformed cells. Genomic DNA exists in the form of chromatin, which generally renders the DNA inaccessible to factors that promote gene expression. Here we report characterization of a cellular protein complex, hSWI/SNF, that can alter chromatin structure in an energy dependent manner and can facilitate the interaction of transcription factors with chromatin. The results indicate that the change in chromatin structure induced by hSWI/SNF is stable in the absence of continued ATP hydrolysis and that the increased ability of transcription factors to bind to chromatin is not dependent on concurrent hSWI/SNF activity, which indicates that hSWI/SNF facilitated binding of transcription factors to nucleosomal DNA is a multi-step process. The results also show that hSWI/SNF can increase transcriptional elongation in vitro on a reconstituted nucleosomal human hsp70 promoter, a promoter that, like c-myc, is regulated at the level of transcription elongation. Additionally, we have demonstrated that in yeast, functional SWI/SNF complex is associated with the RNA pol II holoenzyme. Thus a nucleosome altering activity is associated with the transcription initiation complex, which suggests mechanisms that facilitate stable interaction of the transcription complex with nucleosomal DNA and that facilitate elongation through nucleosomal templates.

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Anthony N. Ambrogiano 10/15/96

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3) Imbalzano, AN, GR Schnitzler, and RE Kingston. 1996. Nucleosome disruption by human SWI/SNF is maintained in the absence of continued ATP hydrolysis. J. Biol. Chem. 271:20726-20733.

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7) Excerpt from NIH RO1 proposal by AN Imbalzano

INTRODUCTION

The objective of this research was to examine regulation of the c-myc proto-oncogene in normal and transformed cells. C-myc has been shown to be overexpressed in a number of human cancers; identifying factors that control c-myc expression and characterizing the mechanisms by which they function should provide insight into how these factors affect normal and transformed cell growth.

In vivo, DNA is complexed with histones and nonhistone proteins to form chromatin, which in general, is refractory to gene expression. Thus to understand how genes such as c-myc become activated, it is necessary to identify and characterize both the in vivo structural state of the promoter controlling gene expression as well as factors that relieve chromatin mediated inhibition and facilitate transcription. Previous work has identified and partially characterized a cellular protein complex called hSWI/SNF that can rearrange chromatin structure in an ATP dependent manner and increase the ability of transcription factors to bind to their recognition sequences when these sequences are incorporated into chromatin (Kwon et al, 1994; Imbalzano et al, 1994). Understanding the mechanism of action of the hSWI/SNF complex is of central importance to analyzing regulation of c-myc and other gene expression. The hSWI/SNF complex has been shown to alter chromatin structure and to facilitate binding of transcription factors to nucleosomal DNA (Imbalzano et al, 1994; Kwon et al, 1994), and individual components of the complex can enhance stimulation of transcription by nuclear hormone receptors in eukaryotic cells (Muchardt and Yaniv, 1993; Khavari et al, 1993) and can bind to the retinoblastoma (Rb) oncoprotein (Dunaief et al, 1994; Singh et al, 1995). The interaction with Rb has been implicated in enhancing nuclear receptor stimulated transcription (Singh et al, 1995) and in causing arrest of the cell cycle (Dunaief et al, 1994). Another component of the complex has been shown to bind to HIV-1 integrase and stimulate DNA joining in vitro, suggesting that the component may promote integration of viral DNA into the genome (Kalpana et al, 1994). In addition, the complex and at least some of the component genes are evolutionarily conserved. Yeast SWI/SNF complex has similar nucleosome altering capabilities (Cote et al, 1994), and the yeast SWI and SNF proteins are required for mating type switching and for the activation of many yeast genes (reviewed in Carlson and Laurent, 1994). Similarly, a Drosophila SWI homologue is required for activation of many homeotic genes and thus for proper development of the organism (Tamkun et al, 1992). Thus SWI/SNF and its components globally affect gene expression, chromatin structure, and cell growth and division, development and differentiation, and potentially are involved in or affect events leading to oncogenesis.

BODY

I. SUMMARY

Before commencing work on a specific promoter like c-myc, our original goal was to complete a thorough characterization of hSWI/SNF mechanism of action and its role in transcription elongation on nucleosomal templates in vitro. This took longer than anticipated. In the first year of the grant, significant progress was made in addressing these topics. In the second year of the grant, time was expended to complete and publish these studies and to pursue other lines of experimentation that were developed from our results as well as from the results of others. Of the other lines of experimentation, one generated results that have been published and two were negative or inconclusive. The fourth is in progress and is the basis for a part of an NIH proposal I have recently submitted. All results are discussed in detail below.

The work accomplished during this grant period significantly advances our understanding of how chromatin structure is altered and made accessible to transcription factors and the general transcription machinery during gene activation. These findings are general in nature and we believe they will be applicable to the regulation of many genes. The work has resulted in three publications and one review article, with the possibility that the work in progress will generate one or more additional publications.

II. PROGRESS ON SPECIFIC AIM #2: In vitro analysis of c-myc promoter occupancy and chromatin structure

A. Characterization of hSWI/SNF activity

1. Experimental Background To characterize the mechanism of hSWI/SNF action, I have employed nucleosomal reconstitution techniques to create rotationally phased nucleosomes in vitro and have the examined cleavage of nucleosomal DNA by DNAse I as a measure of the ability of hSWI/SNF to alter nucleosome structure. DNAse I can only cleave nucleosomal DNA at the point of the DNA helix that is farthest away from the histone core, or in other words, it cleaves the nucleosomal DNA once every turn of the helix, or approximately every 10 base pairs (bp) per strand. Because the assembled mononucleosomes are phased (*ie*- the DNA and the histone core that comprise the nucleosome are physically associated in exactly the same manner for every molecule in the population) and the DNA is end labeled with ^{32}P on one strand only, limiting digestion of the mononucleosomes by DNAse I results in the appearance of a cleavage ladder whose products are 10 bp apart (Imbalzano et al, 1994 and see Figure 1, lane 2, Imbalzano et al, 1996 (appendix 3)). Addition of the hSWI/SNF complex in the presence of ATP results in a decrease in the intensity of the bands forming the 10 bp ladder and the appearance of novel cleavage products in between (see Figure 1, lanes 4-6, Imbalzano et al, 1996 (appendix 3)). Thus the activity of hSWI/SNF alters the nucleosome structure in a manner that allows increased access to the DNAse I enzyme.

2. Results

a) hSWI/SNF activity is dependent on the presence of a hydrolyzable adenosine

triphosphate (ATP). Deoxy-adenosine triphosphate (dATP) could substitute for ATP, however, when dATP was used, a 10-fold higher concentration was required for nucleosome disruption to occur. The nonhydrolyzable ATP analogs ATP-γ-S and AMP-PNP did not support nucleosome disruption, nor did adenosine diphosphate (ADP), nor any other nucleotide triphosphate (Figure 1, Imbalzano et al, 1996 (appendix 3)).

b) ATP is not continuously required for hSWI/SNF activity; the hSWI/SNF mediated alteration in nucleosome structure is stable. If ATP were continuously required for activity, that is, if the structural change induced by hSWI/SNF were transient, and the nucleosome reverted to its original form after ATP mediated disruption, then removal of ATP from the reaction prior to DNAse I cleavage should generate the same 10 bp ladder of cleavage products seen when untreated nucleosomes are digested with DNAse I. Alternately, if the change in structure induced by hSWI/SNF is stable, then the altered DNAse I digestion pattern should appear, even if ATP is removed from the reaction. When hSWI/SNF and nucleosomes were mixed in the presence of ATP and were subsequently exposed to apyrase, which cleaves ATP, or to a 200-fold excess of ATP-γ-S, which is sufficient to inhibit disruption, the altered DNAse I digestion pattern was maintained, indicating that the change in nucleosome structure induced by hSWI/SNF is stable (Figures 2 and 3, Imbalzano et al, 1996 (appendix 3)). This observation was confirmed using nucleosomal plasmid DNA (Figure 4, Imbalzano et al, 1996 (appendix 3)). hSWI/SNF treatment of nucleosomal plasmid DNA results in a reduction in supercoiling; the altered supercoiling pattern was maintained when apyrase or ATP-γ-S was added after hSWI/SNF and ATP were mixed with nucleosomal plasmid DNA. Thus the alteration of chromatin structure caused by hSWI/SNF is stable on both mononucleosome and nucleosomal plasmid DNA.

c) Nucleosome disruption by hSWI/SNF requires the simultaneous presence of nucleosomes and ATP. To determine whether ATP was inducing a conformational change in hSWI/SNF structure that made it "active" and therefore unaffected by the removal of ATP, an order of addition experiment was performed in which hSWI/SNF was mixed with ATP, was subsequently treated with apyrase to remove ATP, and then was mixed with the nucleosomes. In this experiment, the 10 bp ladder of DNAse I cleavage products was observed Figure 5, lane 10, Imbalzano et al, 1996 (appendix 3)), indicating that nucleosome disruption required the simultaneous presence of hSWI/SNF, ATP, and nucleosomes. This result suggests that the hydrolysis of ATP is required for altering the structure of the nucleosome and not for modifying the hSWI/SNF (eg- by phosphorylation, by altering conformation). This work does not exclude the possibility that ATP modifies hSWI/SNF structure, but it indicates that even if such a modification occurs, it is not sufficient for nucleosome disruption.

d) Facilitated binding of the GAL4-AH transcription factor to mononucleosomes containing a GAL4 binding site requires prior disruption by hSWI/SNF but does not

require concurrent activity. Previous work has shown that if the nucleosome contains a transcription factor binding site, treatment of the nucleosome with hSWI/SNF and ATP significantly increases the ability of the transcription factor to bind (*eg*- GAL4 derivatives) or facilitates binding where no binding was previously observed (*eg*- TATA binding protein) (Kwon et al, 1994; Imbalzano et al, 1994). Addition of apyrase to reactions containing nucleosomes and ATP prior to addition of hSWI/SNF prevented hSWI/SNF-mediated nucleosome disruption and prevented facilitated binding of GAL4-AH to the nucleosome (Figure 6- lanes 11-14, Imbalzano et al, 1996 (appendix 3)). When apyrase was added to reactions containing nucleosomes and ATP after the addition of hSWI/SNF, nucleosome disruption was observed, and upon addition of GAL4-AH, facilitated binding of the transcription factor to the altered nucleosome was observed (Figure 6- lanes 15-18, Imbalzano et al, 1996 (appendix 3)). These results indicate that the increased ability of the transcription factor to bind to nucleosomal DNA is not dependent on concurrent hSWI/SNF activity and that hSWI/SNF facilitated binding of transcription factors to nucleosomal DNA can be a multi-step process, where first the nucleosome structure is stably altered by hSWI/SNF, thereby facilitating subsequent interaction with the transcription factor.

e) hSWI/SNF may stably associate with the nucleosome. The observation that hSWI/SNF stably alters nucleosome structure suggested that perhaps hSWI/SNF was stably associated with the altered nucleosome. Preliminary sedimentation studies indicate that when hSWI/SNF and nucleosomes are mixed and subsequently sedimented on a glycerol gradient, the nucleosomes have a significantly increased mobility in the gradient. This change in mobility is apparently not dependent on ATP, although it appears that the presence of ATP slightly changes the increase in migration (Appendix 1- pg. 19). Future efforts are designed to show that the increase in sedimentation is due to a specific interaction between hSWI/SNF and the nucleosome, and to determine the effect of ATP on the interaction.

B. Examination of the ability of hSWI/SNF to stimulate transcription elongation from a nucleosomal human hsp70 promoter.

1. Experimental Background In collaboration with Steven Brown, a graduate student in the lab, investigations into the role of hSWI/SNF in transcription elongation in chromatin were performed (Brown et al, 1996). Steven has shown that in vivo, there is a paused, transcriptionally engaged RNA polymerase II molecule associated with the human heat shock protein 70 (hsp70) promoter, even in the absence of heat shock, when the locus is transcriptionally inactive (Figure 1, Brown et al, 1996 (appendix 4)). To mechanistically examine how activation of the hsp70 promoter occurs upon heat shock, Steven has recreated the inactivated promoter in vitro, establishing a nucleosomal template that has a paused, transcriptionally engaged RNA polymerase molecule and a nascent RNA transcript of the same length that appears in vivo (Figure 2, Brown et al, 1996 (appendix 4)).

2. Results

a) Human heat shock factor activation domain and fractions containing hSWI/SNF

cooperate to increase transcriptional elongation on nucleosomal human hsp70 promoters. The activator that stimulates hsp70 transcription in vivo is the heat shock factor (HSF). Addition of an activator that contains the HSF activation domains to the in vitro system resulted in a small increase in the amount of transcriptional elongation through the pause (fusion proteins consisting of the HSF activation domains and the GAL4 DNA binding domains were used in these experiments because the magnesium and detergent requirements to maintain native human HSF isolated from heat-shocked nuclei active are not compatible with nucleosome assembly conditions). When hSWI/SNF was also added to the reaction, there was a significant increase in the amount of transcriptional elongation, suggesting that addition of a factor able to disrupt nucleosome structure facilitated HSF activation of transcriptional elongation. There was no effect on elongation through the pause by hSWI/SNF in the absence of activator, and activators containing just the GAL4 DNA binding domain had a minimal effect on elongation (Figure 5, Brown et al, 1996 (appendix 4)). Quantification of elongated product at different hSWI/SNF concentrations is presented in Figure 6, Brown et al, 1996 (appendix 4). The data showing stimulation of elongation on nucleosomal DNA by hSWI/SNF in conjunction with the HSF activation domain provides the first demonstration that a chromatin remodeling activity (hSWI/SNF) has an effect on transcriptional activation.

b) GAL4-HSF and hSWI/SNF do not affect elongation from the paused polymerase on naked hsp70 DNA. Examination of elongation on naked hsp70 templates revealed some short-lived pause sites, however, these were unchanged in the presence of GAL4-HSF and/or hSWI/SNF (Figure 7, Brown et al, 1996 (appendix 4)).

C. Can activators directly target hSWI/SNF to DNA?

Experimental Background and Results

I had previously shown that hSWI/SNF could increase the ability of GAL4 activators to bind to nucleosomal DNA in an ATP dependent manner, and that a GAL4 activator with a strong activation domain bound with greater affinity than did an activator with a weaker or no activation domain. No effect of the activation domain on binding to naked DNA or binding to nucleosomal DNA in the presence of hSWI/SNF without ATP was observed (Kwon et al, 1994). This suggested that the activation domain affects the ability of the activator to bind to nucleosomes altered by hSWI/SNF. To determine whether GAL4 activators could directly target hSWI/SNF to nucleosomal DNA, I added increasing amounts of hSWI/SNF to nucleosomes containing a GAL4 binding site (in the presence of ATP) and determined the concentration of hSWI/SNF required to alter the DNAse I digestion pattern. I then repeated the experiment in the presence of GAL4 activators containing either a strong, a weak, or no activation domain. If the activator could target hSWI/SNF, then one would predict that the alteration of the nucleosomal DNA would occur at lower concentration of hSWI/SNF, since targeting by the activator would increase the local concentration at the nucleosome. No differences in amount of hSWI/SNF required were observed in the presence of any of the activators. Activator

concentration was varied such that the activator:hSWI/SNF ration ranged from 1:1 to 100:1 without effect. One interpretation is that activators do not directly target hSWI/SNF to nucleosomes. Alternately, the correct conditions may not have been achieved to see targeting. One concern was that in this assay, the nucleosome concentration was very low, and raising the nucleosome concentration significantly is not possible due to the dilution that occurs when the nucleosomes are gradient purified. Other experimental approaches to addressing this question have not been pursued.

D Yeast RNA polymerase II holoenzyme contains SWI/SNF, can alter nucleosome structure, and facilitates TBP binding to nucleosomal DNA.

1. Experimental Background

In yeast, it has been proposed that in vivo, the RNA pol II exists in a complex called the holoenzyme that also contains several of the pol II general transcription factors (GTFs) and a group of proteins called SRB proteins, which form a complex that is in association with the large subunit C terminal repeat domain and which are part of a mediator complex that helps stimulate transcription by activator proteins. In addition, holoenzyme contains a number of unidentified proteins. Richard Young's group at MIT made the observation that the yeast SWI/SNF proteins are part of the holoenzyme as well as part of the SRB subcomplex (Figures 1-5, Wilson et al, 1996 (appendix 5)). This was demonstrated by showing that SWI and SNF proteins co-purified with SRB and other holoenzyme components over every purification step. In addition, immunoprecipitation experiments showed that SWI and SNF proteins could be immunoprecipitated from purified or crude holoenzyme (or SRB) preparations with antibodies against SRB proteins, and conversely, SRB proteins could be immunoprecipitated by antibodies against a SWI/SNF component.

2. Results

a) Holoenzyme and the SRB complex alter nucleosome structure in an ATP dependent manner Fractions containing pol II holoenzyme or purified SRB complex were mixed with rotationally phased mononucleosome particles in the presence or absence of ATP and subsequently digested with DNAse I. An ATP dependent alteration in the DNAse I digestion pattern was observed, indicating that both complexes were able to alter nucleosome structure (Figure 6, Wilson et al, 1996 (appendix 5)) . Further experiments demonstrated that both the holoenzyme and SRB complex could reduce the supercoiling of nucleosomal plasmid DNA, confirming the ability to alter chromatin structure in an ATP dependent manner (Figure 7, Wilson et al, 1996 (appendix 5)).

b) Holoenzyme facilitates binding of the TATA binding protein (TBP) and TFIIA to a nucleosomal template containing a specifically oriented TATA box. Yeast RNA polymerase II holoenzyme contains several general transcription factors (GTFs), but does not contain TBP or TFIIA, which are the first factors to associate with the TATA box during transcription complex assembly on a promoter. Since the holoenzyme possessed the ability to alter nucleosome structure and since hSWI/SNF had been shown to facilitate binding of TBP and TFIIA to a nucleosomal template containing a

specifically oriented TATA box (ie- the binding site was in a fixed spot in the DNA helix relative to the surface of the histones- Imbalzano et al, 1994), I asked whether holoenzyme could similarly facilitate TBP/TFIIA binding. Nucleosome particles were mixed with ATP in the presence or absence of holoenzyme, and, subsequently, TBP and TFIIA were added. The reactions were then digested with DNAse I, and resolution of the digestion products indicated that in the presence of holoenzyme, TBP/TFIIA protected the nucleosome from DNAse I digestion directly over the TATA box (Figure 8A, Wilson et al, 1996 (appendix 5)). Thus TBP/TFIIA were specifically bound to the holoenzyme altered nucleosome. Binding occurred with a K_D of approximately 4×10^{-6} M. This value is similar to the K_D exhibited by TBP/TFIIA for this template in the presence of hSWI/SNF, and indicates that affinity of TBP/TFIIA in the presence of either hSWI/SNF or holoenzyme is reduced by over 100-fold relative to the affinity of TBP/TFIIA for naked DNA.

c) Holoenzyme requires ATP to alter nucleosome structure, however, facilitated binding of TBP/TFIIA is enhanced by, but does not require, ATP Although the alteration of nucleosome structure required ATP, holoenzyme could facilitate binding by TBP/TFIIA in the absence of ATP and nucleosome disruption. Mixture of nucleosomes with holoenzyme in the absence of ATP revealed no significant change in the nucleosome structure and partial protection of the TATA box upon DNAse I digestion (Figure 8B, Wilson et al, 1996 (appendix 5)). The same partial protection and absence of change in nucleosome structure was observed in the presence of ATP-γ-S. In the presence of ATP, however, nucleosome structure was altered, as demonstrated by the change in the DNAse I digestion pattern, and the protection over the TATA box was enhanced and was extended in the 5' direction.

d) Holoenzyme facilitates binding of TBP/TFIIA in a manner that is independent of the orientation of the binding site on the nucleosome. My previous work has demonstrated that human SWI/SNF could facilitate binding of TBP and TFIIA to a nucleosomal template containing a specific orientation of the TATA box, but not to templates that had the TATA box in different orientations (Imbalzano et al, 1994). Thus the ability to facilitate binding is dependent upon the position of the binding site on the nucleosome. This (and other data) indicate that the histones are still present on the altered nucleosome and strongly suggests binding is limited to orientations of the TATA box that can accommodate both the bend in the DNA due to association with the histones as well as the bend induced in the DNA by TBP binding (Imbalzano et al, 1994). To determine whether the ability of holoenzyme to facilitate TBP/TFIIA binding was also influenced by the position of the binding site, nucleosomes containing TATA boxes in different orientations were assembled and mixed with ATP in the presence or absence of holoenzyme. Holoenzyme facilitated binding of TBP/TFIIA to all three orientations tested (appendix 2- pg. 20), indicating that the activity of holoenzyme was sufficient to overcome restrictions in binding of TBP/TFIIA due to orientation of the TATA box. However, the K_D for TBP/TFIIA binding remained in the 10^{-6}-10^{-7} M range, raising the possibility that additional factors are required for high affinity binding of the GTFs to nucleosomal DNA.

e) Implications for transcriptional regulation The association of functional SWI/SNF with the RNA polymerase holoenzyme provides many new models for how a number of steps in the process of transcription initiation and elongation may occur. Many transcriptional activators that bind to upstream promoter sequences can physically contact GTFs that associate with polymerase II; it has long been postulated that activators may target the GTFs and/or polymerase to the promoter. Evidence that an activator can target pol II holoenzyme on naked DNA was recently published (Barberis et al, 1995). If SWI/SNF is associated with the polymerase holoenzyme, then a putative mechanism by which SWI/SNF can be brought to the promoter is via activator interactions with the basal machinery (see Figure 2, Kingston et al, 1996, appendix 6)). Competition between transcription factors and chromatin proteins could be resolved by recruitment of holoenzyme to the promoter if SWI/SNF mediated alterations in local chromatin structure promoted a more stable interaction between the transcription factors and their binding sites. In addition, the ability of holoenzyme to facilitate binding of TBP/TFIIA in the absence of ATP raises the possibility that the polymerase associated proteins have an intrinsic ability to function in chromatin, without the activity of SWI/SNF. One prediction is that if there is strong recruitment of holoenzyme by the activator, TBP/TFIIA binding could be facilitated by the relatively high concentration of holoenzyme at the promoter. In contrast, if targeting by a different activator is weak, or if there is a refractory chromatin structure at the promoter, the nucleosome altering activity of SWI/SNF could promote TBP/TFIIA binding. This might explain why some but not all promoters in yeast require SWI/SNF. Finally, the localization of a nucleosome altering activity with the polymerase at the start site of transcription has interesting implications for elongation though nucleosomal templates. One prediction is that the SWI/SNF remains associated with the polymerase during elongation and functions to alter nucleosome structure so that elongation through nucleosomes is facilitated. The work presented in part (B) above shows that addition of hSWI/SNF to a paused polymerase significantly enhances the ability of an activator to promote transcriptional elongation. This suggests that nucleosome disruption contributes to efficient elongation, and supports the hypothesis.

E. Does the Rb oncoprotein affect hSWI/SNF mediated nucleosome disruption?
Experimental Background and Results

Some components of hSWI/SNF can bind to the retinoblastoma (Rb) oncoprotein (Dunaief et al, 1994; Singh et al, 1995). The interaction with Rb has been implicated in enhancing nuclear hormone receptor stimulated transcription (Singh et al, 1995) and in causing arrest of the cell cycle (Dunaief et al, 1994). To determine whether the potential for interaction with Rb would affect the ability of hSWI/SNF to alter chromatin structure, the effects of adding purified Rb to nucleosome disruption assays were examined. Purified recombinant Rb was obtained from B. Dynlacht and E. Harlow (Massachusetts General Hospital) and was mixed with hSWI/SNF in the presence or absence of ATP, before addition of nucleosomes. Additionally, Rb was added to reactions in which hSWI/SNF had already been mixed with nucleosomes (+/- ATP). No effect on alteration of nucleosomal DNA by hSWI/SNF was observed. One interpretation is that Rb does not affect the nucleosome altering function of

hSWI/SNF, however, no definitive conclusions could be reached because it is possible that the Rb was not active enough, was not present at a high enough concentration, was not properly modified (eg- by phosphorylation), or required some other factor or condition for function to be observed.

III. PROGRESS ON SPECIFIC AIM #1:

A. In vivo analysis of c-myc promoter occupancy and chromatin structure.

Experimental Design and Results

Mapping of c-myc promoter structure was proposed by chemical or enzymatic modification of genomic DNA in vivo, with subsequent detection of DNA cleavage sites by amplification with ligation mediated polymerase chain reaction (LMPCR). Due to the focus on further characterization of hSWI/SNF, only a few attempts to initiate in vivo mapping of c-myc promoter structure were made during the first year of the grant; no signal was observed, indicating that optimization of LMPCR reaction conditions and/or different LMPCR primers was required. No further progress on this topic has been made.

B. Alternate experimental approach: Determination of the effects of hSWI/SNF components on activation of gene expression and on differentiation processes in vivo

Experimental Background

In mammalian cells there are two closely related homologues of the yeast SWI2 protein, which is the ATP binding subunit of the yeast SWI/SNF complex. Both of these proteins, called BRG1 and hBRM, are present in our hSWI/SNF preparations, although it is not clear whether there is a mixture of complexes comprising the hSWI/SNF activity or there is one complex that contains both BRG1 and hBRM. When BRG1 or hBRM was transfected into eukaryotic cells with a reporter plasmid, activation of transcription by nuclear hormone receptors is enhanced (Khavari et al, 1993; Muchardt and Yaniv, 1993; Chiba et al, 1994). When plasmids encoding BRG1 or hBRM proteins that are mutated in the ATP binding site were used in these experiments, activation of the reporter gene by the nuclear hormone receptors was inhibited or reduced (Khavari et al, 1993; Muchardt and Yaniv, 1993). Thus these mutant BRG1 and hBRM proteins act as dominant negatives, in that they interfere with the ability of the wild-type protein to function. To identify gene activation events and cellular processes that are affected by hSWI/SNF components/complexes, I am creating cells that inducibly express the dominant negative proteins. The premise is that if BRG1 or hBRM are involved in a specific regulatory event, expression of the dominant negative protein will affect that event.

Conditional expression will be achieved using the Tet repressor system established by Gossen and Bujard (Gossen and Bujard, 1992), with the modifications of Shockett et al (Shockett et al., 1995). Briefly, cells will first be stably transformed with a vector that encodes a Tet-VP16 regulatory protein, which is a fusion of the DNA binding domain of the Tet repressor with the activation domain of the herpes simplex virus activator VP16, and a vector encoding a gene for puromycin resistance,

for selection purposes. The fusion protein is active in the absence of tetracycline or at low tetracycline concentrations and can therefore stimulate expression from promoters containing a binding site for Tet repressor. Since the Tet repressor is inactivated (unable to bind DNA) at high tetracycline concentrations, expression of promoters containing a Tet repressor binding site is shut off when high concentrations of tetracycline are added to the media.

Currently, cell lines under construction are derived from HeLa and NIH 3T3 cells. Both lines now contain the Tet-VP16 regulator and I am in the process of isolating lines that inducibly express dominant negative BRG1 or hBRM (4 lines total). When completed, the 3T3 lines will be used to examine the role of hSWI/SNF proteins in differentiation of muscle cells and in differentiation of fat cells. In Drosophila, a homologue of one of the SWI/SNF components is involved in the regulation of homeotic gene expression and is required for proper development of the organism (Tamkun et al, 1992). Previous work has shown that upon expression MyoD and removal of serum from the media, 3T3 cells can be induced to differentiate into muscle-like cells that express many muscle specific genes (Davis et al, 1987). To determine whether there is an effect on differentiation due to BRG1 or hBRM, the dominant negative protein will be induced prior to the start of the differentiation process. Additionally, to determine whether there is a role for BRG1 or hBRM in maintenance of the differentiated state, other experiments will be performed in which differentiation is allowed to occur before production of the dominant negative protein. A failure to maintain the differentiated state would reflect a role for the hSWI/SNF protein(s). Since 3T3 cells can also be induced to differentiate into fat cells by expression of the nuclear hormone receptor PPARγ under differentiation inducing culture conditions (Tontonoz et al, 1994), analogous experiments will be carried out under these conditions to examine a possible role for BRG1/hBRM in adipocyte differentiation.

The HeLa cells will be used by Bob Kingston's lab to characterize any effects of the dominant negatives on heat shock gene expression and by me to examine whether rapid induction of the proto-oncogenes c-myc and c-fos by estrogen and serum, respectively, are affected by the expression of the dominant negatives. Finally, in Jan., 1997, I move to an assistant professor position at the University of Massachusetts Medical Center. There I will use the technology described above to examine activation of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) by glucocorticoids in human breast cancer cells (a copy of the relevant section of the NIH RO1 application I have submitted is enclosed in appendix 7 of this report).

CONCLUSION

Activation of gene expression in vivo occurs in chromatin, yet, in general, chromatin structure inhibits multiple steps in the process of transcriptional activation. Determining how the cell overcomes the repressive effects of chromatin structure is essential to understanding how activation of gene expression occurs and may provide insight into how failure to appropriately regulate gene expression may lead to developmental abnormalities and/or cellular transformation.

During the grant period, significant progress has been made in characterizing an energy dependent cellular activity that alters nucleosome structure and facilitates transcription factor binding to nucleosomal DNA. These studies have had significant impact on the field of transcriptional regulation. Our results have provided the field with a considerable amount of new data that addresses how a number of steps in the process of transcriptional activation might be facilitated by factors that alter nucleosome structure and also have provided a number of novel models for gene activation in chromatin that are being tested by us and others.

Specifically, we have determined that the alteration of nucleosome structure mediated by hSWI/SNF is stable in the absence of continued ATP hydrolysis. Furthermore, facilitated binding of transcription factors to hSWI/SNF altered nucleosomes can occur in the absence of continued hSWI/SNF activity. These results demonstrate that altering chromatin structure and facilitating transcription factor binding can be temporally separated. Thus we have defined potential steps that may occur in the process of gene activation in chromatin. Furthermore, we have demonstrated that hSWI/SNF can enhance activator mediated elongation of transcription from a template containing a paused, transcriptionally engaged RNA polymerase II. This is the first demonstration that a chromatin remodeling activity can facilitate transcription. We have also, in collaboration with Rick Young, demonstrated that the yeast SWI/SNF complex is a functional part of the pol II holoenzyme. Thus a complex consisting of pol II and many of its associated factors also contains a nucleosome altering activity that in vitro can facilitate stable interaction of the basal transcription machinery with nucleosomal DNA. Finally, work in progress is designed to identify in vivo targets for hSWI/SNF activity so that the role of hSWI/SNF in specific gene activation events and/or differentiation processes in higher eukaryotes can be determined.

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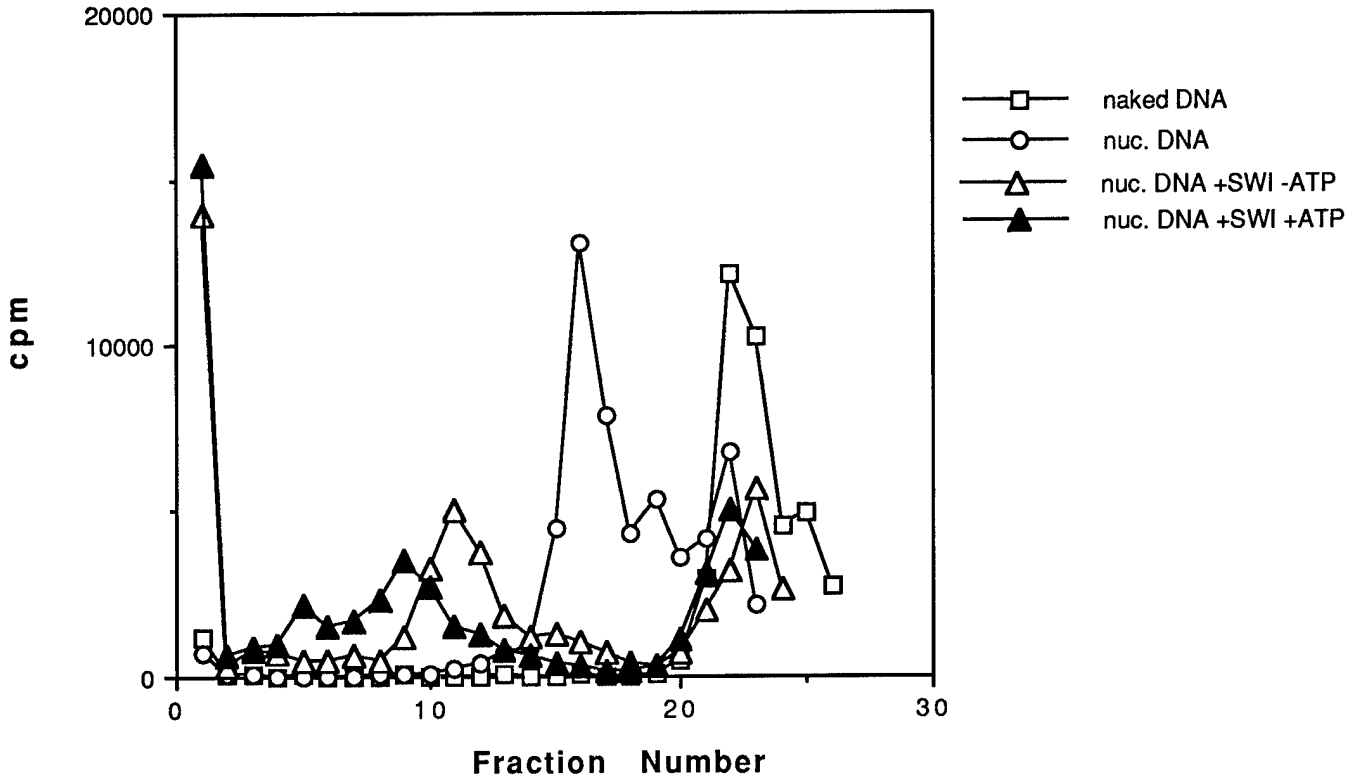
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Figure 1

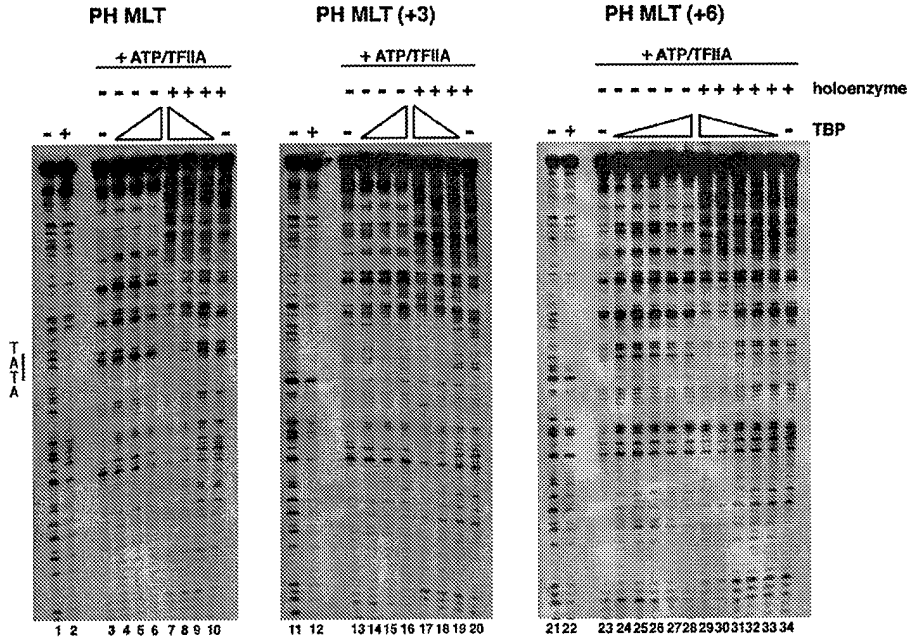
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Figure 2

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Nucleosome Disruption by Human SWI/SNF Is Maintained in the Absence of Continued ATP Hydrolysis*

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We have examined the requirement for ATP in human (h) SWI/SNF-mediated alteration of nucleosome structure and facilitation of transcription factor binding to nucleosomal DNA. hSWI/SNF-mediated nucleosome alteration requires hydrolysis of ATP or dATP. The alteration is stable upon removal of ATP from the reaction or upon inhibition of activity by excess ATPγS, indicating that continued ATP hydrolysis is not required to maintain the altered nucleosome structure. This stable alteration is sufficient to facilitate binding of a transcriptional activator protein; concurrent ATP hydrolysis was not required to facilitate binding. These data suggest sequential steps that can occur in the process by which transcription factors gain access to nucleosomal DNA.

In vivo, DNA is compacted via association with histones and nonhistone proteins to form chromatin, which, in general, inhibits the interaction of the transcriptional machinery with promoter sequences and is therefore refractory to gene expression. While inactive promoters are generally incorporated in nucleosomal arrays, regulatory sequences controlling the expression of genes being actively transcribed have been shown to exist in a more open conformational state, as shown by their increased sensitivity to cleavage by nucleases. Thus, there must be mechanisms utilized by the cell to disrupt chromatin and render relevant DNA sequences accessible to the transcriptional machinery.

A number of different mechanisms may exist to explain how chromatin structure is altered on promoter/enhancer sequences. Many activators, such as Sp1 (Chen *et al.*, 1994; Li *et al.*, 1994), synthetic derivatives of the yeast GAL4 transcriptional activator (Taylor *et al.*, 1991; Workman and Kingston, 1992), progesterone receptor (Pham *et al.*, 1992), glucocorticoid receptor (Perlmann and Wrange, 1988; Pina *et al.*, 1990; Archer *et al.*, 1991; Li and Wrange, 1993; Li and Wrange, 1995), TFIIIA[1] (Rhodes, 1985; Lee *et al.*, 1993), upstream stimulatory factor (Chen *et al.*, 1994; Adams and Workman, 1995), and Max and c-Myc-Max heterodimers (Wechsler *et al.*, 1994) have been shown to bind to nucleosome particles *in vitro*. In some cases, the binding of activators can destablize the nucleosome, as shown by the observation that the binding of GAL4 derivatives to mononucleosome particles containing five GAL4 sites facilitates the displacement of histones to histone acceptors (Workman and Kingston, 1992; Chen *et al.*, 1994; Walter *et al.*, 1995). In other cases, direct modification of the histone proteins comprising the nucleosome can alter the accessibility of the nucleosome to transcription factors. For example, TFIIIA binding to mononucleosomes can be facilitated by acetylation of the N-terminal tails of the core histones (Lee *et al.*, 1993).

Other proposed mechanisms involve activities that mediate energy-dependent chromatin alteration (Cote *et al.*, 1994; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a; Tsukiyama *et al.*, 1994; Tsukiyama and Wu, 1995; Wall *et al.*, 1995; Varga-Weisz *et al.*, 1995; Pazin *et al.*, 1994). Some of these activities are due to large protein complexes (*e.g.* SWI/SNF, NURF) that hydrolyze ATP and structurally alter nucleosome particles (Cote *et al.*, 1994; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a; Tsukiyama and Wu, 1995; Tsukiyama *et al.*, 1995). The yeast SWI/SNF complex is comprised of the products of several yeast *SWI* and *SNF* genes that function together in a large, multisubunit complex (Laurent and Carlson, 1992; Peterson and Herskowitz, 1992; Peterson *et al.*, 1994; Cairns *et al.*, 1994) that is required for the transcription of a large number of inducible genes and has been shown to enhance the function of many yeast, fly, and human transcriptional activators in yeast cells (Laurent and Carlson, 1992; Peterson and Herskowitz, 1992; Yoshinaga *et al.*, 1992; reviewed in Winston and Carlson, 1992). Similarly, two human homologs of the helicase-related ATPase SNF2/SWI2 have been shown to enhance nuclear hormone receptor function in mammalian cells (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994) and to purify as part of a large molecular weight complex (Khavari *et al.*, 1993; Kwon *et al.*, 1994).

Although individual yeast *SNF* genes have been shown to function as activators when fused to DNA binding domains (Laurent *et al.*, 1990; Laurent *et al.*, 1991; Laurent and Carlson, 1992), the SWI/SNF complex appears not to function as an activator or bridging coactivator. Instead, many lines of evidence point to a role in chromatin disruption. First, phenotypes caused by mutations in yeast *SWI* and *SNF* genes can be suppressed by mutations in histone and nonhistone chromatin proteins and by mutations that alter histone expression levels (Kruger and Herskowitz, 1991; Peterson *et al.*, 1991; Hirschhorn *et al.*, 1992; Kruger *et al.*, 1995). SWI/SNF mutations have also been shown to mediate structural changes in chromatin *in vivo* (Hirschhorn *et al.*, 1992; Matallana *et al.*, 1992). More recently, both the yeast and human SWI/SNF complexes have been purified and shown to directly alter nucleosome structure as well as to facilitate transcription factor binding to nucleosomal DNA in an ATP-dependent manner (Cote *et al.*, 1994; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a).

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[1] The abbreviations used are: TF, transcription factor; h, human; bp, base pair(s); ATPγS, adenosine 5′-*O*-(thiotriphosphate); AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; NURF, nucleosome remodeling factor; BSA, bovine serum albumin.

Another clue to the mechanism of SWI/SNF complex function is provided by the observation that active SWI/SNF complex is a component of the yeast RNA polymerase II holoenzyme (Wilson *et al.*, 1996), which is thought to be the form of polymerase responsible for mRNA synthesis *in vivo* (reviewed in Carey, 1995; Emili and Ingles, 1995; Koleske and Young, 1995). This suggests that holoenzyme, perhaps targetted to an inactive promoter by activators capable of weakly binding chromatin, carries with it an activity capable of disrupting repressive chromatin structure and allowing preinitiation complex formation.

Thus, understanding the mechanism by which SWI/SNF complexes alter chromatin structure is likely to be important in determining how transcriptionally inert genes become activated and may be applicable as well to other processes involving utilization of the DNA in chromatin, such as replication and viral integration (Dunaief *et al.*, 1994; Kalpana *et al.*, 1994). To further characterize the mechanism by which the human SWI/SNF (hSWI/SNF) complexes alter chromatin structure, we have investigated the ATP requirement for hSWI/SNF activity as well as the nature of the structural change induced by hSWI/SNF. We report that the change in chromatin structure induced by hSWI/SNF is stable, even in the absence of continued ATP hydrolysis, on both mononucleosome and nucleosomal plasmid templates. In addition, hSWI/SNF-facilitated transcription factor binding to nucleosomal DNA requires nucleosome alteration but does not require concurrent ATP hydrolysis.

MATERIALS AND METHODS

Protein Purification—HeLa cell pellets were obtained from the National Cell Culture Center (Minneapolis, MN). hSWI/SNF "A" and "B" fractions were isolated from HeLa cell nuclear extract according to the method of Kwon *et al.* (1994) through the Econo Q stage. The hSWI/SNF A preparation was 150 ng/μl (determined by Bradford assay) and was estimated to be ~5% pure by comparing silver-stained SDS-polyacrylamide gels to silver-stained gels depicting hSWI/SNF preparations further purified on a Superose column (Kwon *et al.*, 1994). The hSWI/SNF B preparation used in all experiments except that presented in Fig. 4 was 200 ng/μl and was estimated to be ~3% pure. The hSWI/SNF B preparation used in the experiment presented in Fig. 4 was 90 ng/μl and was estimated to be ~10% pure. The estimates of purity were corroborated by activity assays showing that each complex is able to fully alter nucleosome structure at an apparent stoichiometry of 1:1 when calculations are based on these estimates. GAL4-AH (*i.e.* GAL4-(1–147)-amphipathic helix) was purified as described (Lin *et al.*, 1988). HeLa core histone octamers were purified as described (Workman *et al.*, 1991b).

Mononucleosome Assembly—Mononucleosome particles were assembled by salt dilution as described (Imbalzano *et al.*, 1994a) using PH MLT, PH MLT(+3), or PH $GAL4_1$ 150 bp, gel-purified restriction fragments (Imbalzano *et al.*, 1994a; Kwon *et al.*, 1994). The GAL4 site in PH $GAL4_1$ is the synthetic consensus site CGGAAGACTCTCCTCCG defined by Giniger *et al.* (1985). Assembly reactions contained 0.45 μg of ^{32}P-labeled DNA fragment (end-labeled by Klenow fill-in with [^{32}P]dATP (DuPont NEN) at the *Eco*RI end or with [^{32}P]dCTP (DuPont NEN) at the *Bam*HI end), 5 μg of *Hae*III-digested pUC18 DNA, and 8.6 μg of core histone octamers purified from HeLa cell nuclei (Workman *et al.*, 1991b). The concentration of core histone octamers was determined by Bradford assay using BSA as a standard. Mononucleosomes were separated from unincorporated DNA on 5-ml 5–30% glycerol gradients containing 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mg of BSA per ml in a Beckman SW55.1 rotor spun at 35,000 rpm at 4 °C for 15 h. 2 μl from each gradient fraction were counted by scintillation counting; 3 μl were subjected to electrophoresis on a native 5% polyacrylamide gel containing either 1 or 0.5 × TBE (TBE, Tris/borate/EDTA) to monitor assembly. The nucleosome concentration of peak fractions, including nucleosomes assembled onto unlabeled carrier DNA, was typically 0.8 to 4 ng/μl. Fractions were stored at 4 °C.

DNase Reaction Conditions—0.3 ng (1.2×10^{-10} M) of labeled nucleosomes (approximately 3 ng (1.2×10^{-9} M) total nucleosomes) were added to reactions of 25 μl of total volume that contained 12 mM HEPES, pH 7.9, 60 mM KCl, 7 mM $MgCl_2$, 15% glycerol, 0.5 μg of BSA, and 10 mM Tris-HCl, pH 7.5 (contributed from the glycerol gradient buffer), 0.6 mM dithiothreitol, 0.06 mM EDTA. Where indicated, reactions contained ATP (Sigma), the nonhydrolyzable ATP analogs ATPγS or AMP-PNP (Sigma), UTP, CTP, GTP (Pharmacia Biotech Inc.), or ADP (Boehringer Mannheim) at concentrations from 0.02 to 4 mM; see figure legends for each experiment. Apyrase (Sigma) was resuspended in 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 mg of BSA per ml at a concentration of 1 unit/μl. Reactions were incubated at 30 °C for the times indicated in each figure legend.

Following incubation, reactions were treated with 0.01 unit (for reactions containing naked DNA) or 0.1 unit (for reactions containing nucleosomal DNA) of RQ1 DNase I (Promega) for 2 min at room temperature. Digestion was stopped by addition of 2 μl of 0.5 M EDTA. Samples were prepared for electrophoresis on 8% polyacrylamide sequencing gels as described (Imbalzano *et al.*, 1994b).

Assembly of Nucleosomal Plasmid Templates and Supercoiling Assay—pG_5HC_2AT (Workman *et al.*, 1991a), a 3.35-kilobase plasmid, was linearized with *Bam*HI, labeled with [γ-^{32}P]ATP (DuPont NEN) and T4 polynucleotide kinase (New England Biolabs), extracted with phenol:chloroform (1:1), purified through a Sephadex G-50 spin column (Pharmacia), equilibrated in TE (TE, Tris/EDTA), and re-ligated at a concentration of 1 μg/ml. The closed circular, internally labeled plasmid was reconstituted as described with purified HeLa core histone octamers in a *Xenopus* heat-treated extract that is competent for nucleosome assembly (Workman *et al.*, 1991b), in the presence of wheat germ topoisomerase I (Promega). The reconstituted plasmids were purified on 5-ml 10–40% glycerol gradients in a Beckman SW55.1 rotor spun at 35,000 rpm at 4 °C for 4 h.

Reactions contained nucleosomal template (1–2 ng of DNA), 1 unit of topoisomerase I (Promega), 2.5 μl of 30% glycerol gradient buffer, 4 mM $MgCl_2$ and, where indicated, hSWI/SNF, 0.4 mM ATP, 7 mM ATPγS/$MgCl_2$, and/or 2.5 units of apyrase, and were brought to a final volume of 12.5 μl with Buffer A (20 mM HEPES, pH 7.9, 100 mM KCl, 10% glycerol, 0.2 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). Except where noted, reactions were incubated for 90 min at 30 °C and then stopped with 6 μl of stop buffer (3% SDS, 100 mM EDTA, 50 mM Tris, pH 8.0, 25% glycerol, 2 μg/μl proteinase K). Reactions were then incubated at 37 °C for 90 min and resolved on 2% agarose, 50 mM Tris phosphate, pH 7.3, 1 mM EDTA gels in the absence or presence of 0.5 μM chloroquine for 40 h at 40 V. Gels were then dried and exposed to film.

Assessment of Nucleosome Stability—Identical reactions containing naked or nucleosomal PH MLT or PH MLT(+3) DNA were set up and incubated at 30 °C under the reaction conditions described above, in the absence or presence of 4 mM ATP. At the times indicated, reactions were removed from the 30 °C bath and loaded directly onto adjacent lanes of native 5%, 0.5 × TBE polyacrylamide gels that had been pre-run at 4 °C for 1 h. Electrophoresis was performed at 6 V/cm. Gels were then dried and exposed to film and to a phosphorimager screen (Molecular Dynamics). The proportion of free DNA present in the nucleosomal lanes was quantified by the calculation: volume free DNA band/(volume nucleosomal DNA band + volume free DNA band).

RESULTS

To investigate the requirements for and to assess changes in nucleosome structure due to hSWI/SNF activity, rotationally phased nucleosome particles were assembled *in vitro* from ^{32}P-end-labeled, gel-purified 150-bp DNA fragments and purified HeLa cell histone octamers. Mononucleosome particles were separated from unassembled DNA by glycerol gradient centrifugation. The purified mononucleosome particles showed decreased mobility relative to naked DNA on native polyacrylamide gels (see below), were resistant to micrococcal nuclease digestion (Imbalzano *et al.*, 1994a), and exhibited a 10-bp cleavage ladder upon digestion with DNase I (Fig. 1), which is typical of a mononucleosome population that is rotationally phased.

Nucleotide Requirements for hSWI/SNF Function—Previous work has demonstrated that hSWI/SNF-mediated nucleosome disruption requires ATP and is not promoted by nonhydrolyzable ATP analogs (Kwon *et al.*, 1994). To further investigate the nucleotide requirements for nucleosome disruption, ATP was replaced by different nucleoside di- or triphosphates, and concentrations ranging from 20 μM to 2 mM of each nucleotide were tested (Fig. 1). hSWI/SNF activity resulted in a decrease in the

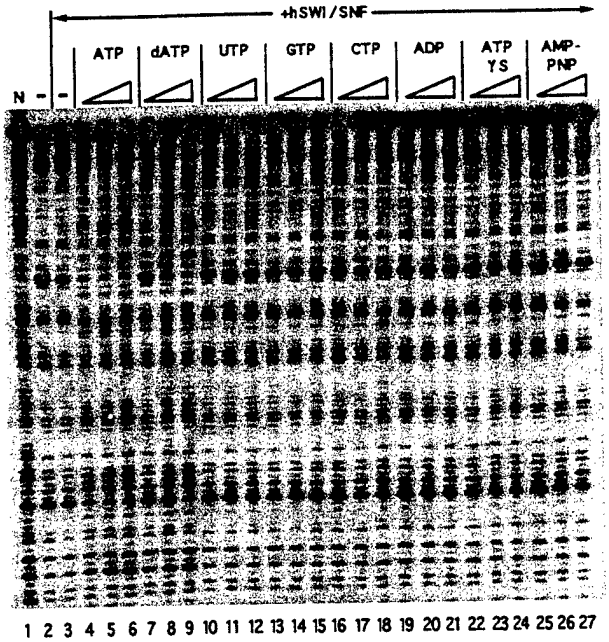

FIG. 1. **ATP and dATP support nucleosome disruption by hSWI/SNF.** Reactions contained 0.3 ng (1.2×10^{-10} M) of PH MLT nucleosomes labeled at the *Eco*RI site (approximately 3 ng (1.2×10^{-9} M) total nucleosomes) in 25-μl reactions containing, where indicated, 600 ng of hSWI/SNF B fraction (*lanes 3–27*). Where indicated, reactions also contained 0.02 mM (*lanes 4, 7, 10, 13, 16, 19, 22, 25*), 0.2 mM (*lanes 5, 8, 11, 14, 17, 20, 23, 26*), or 2 mM (*lanes 6, 9, 12, 15, 18, 21, 24, 27*) nucleoside tri- or diphosphate or nonhydrolyzable ATP analog. Reactions were incubated at 30 °C for 30 min, followed by DNase I digestion. *N* represents naked DNA.

intensity of the cleavage products comprising the 10-bp repeat pattern and the appearance of novel cleavages throughout the length of the template. 20 μM ATP was almost as effective as 2 mM ATP at eliciting maximal activity (*lanes 4–6*; similar results were seen at lower amounts of hSWI/SNF, data not shown). This change in accessibility to DNase I indicates that hSWI/SNF mediates an ATP-dependent alteration in the structure of the nucleosomal DNA (compare *lanes 3* and *4*). *Lanes 22–27* confirm that ATP hydrolysis is required for hSWI/SNF function as neither ATPγS nor AMP-PNP, both nonhydrolyzable ATP analogs, supported nucleosome disruption. Of the analogs tested, only dATP could substitute for ATP, although approximately 10-fold more dATP than ATP was required to see disruption (*lanes 7–9*). UTP, GTP, CTP, and ADP did not promote disruption (*lanes 10–21*).

Prior work has demonstrated that two chromatographically separable fractions that contain hSWI/SNF nucleosome disruption activity can be obtained from fractionation of HeLa cell nuclear extract. These fractions were termed A and B (Kwon *et al.*, 1994). Both contained immunoreactivity to BRG1, a SWI2/SNF2 homolog (Khavari *et al.*, 1993), and they showed no functional differences in all previous studies (Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a). Fig. 2*A* demonstrates that the alteration in the DNase I digestion pattern of mononucleosomes mixed with either hSWI/SNF A or hSWI/SNF B is the same. Both hSWI/SNF A and hSWI/SNF B were used separately in all of the experiments presented in this paper, and essentially identical results were obtained for each experiment (data not shown). The fact that all results are observed with both SWI/SNF complexes argues that they are unlikely to be caused by a fortuitously co-purifying activity because the hSWI/SNF A and B fractions are both highly enriched and they chromatograph differently on both phosphocellulose and single strand DNA-cellulose columns.

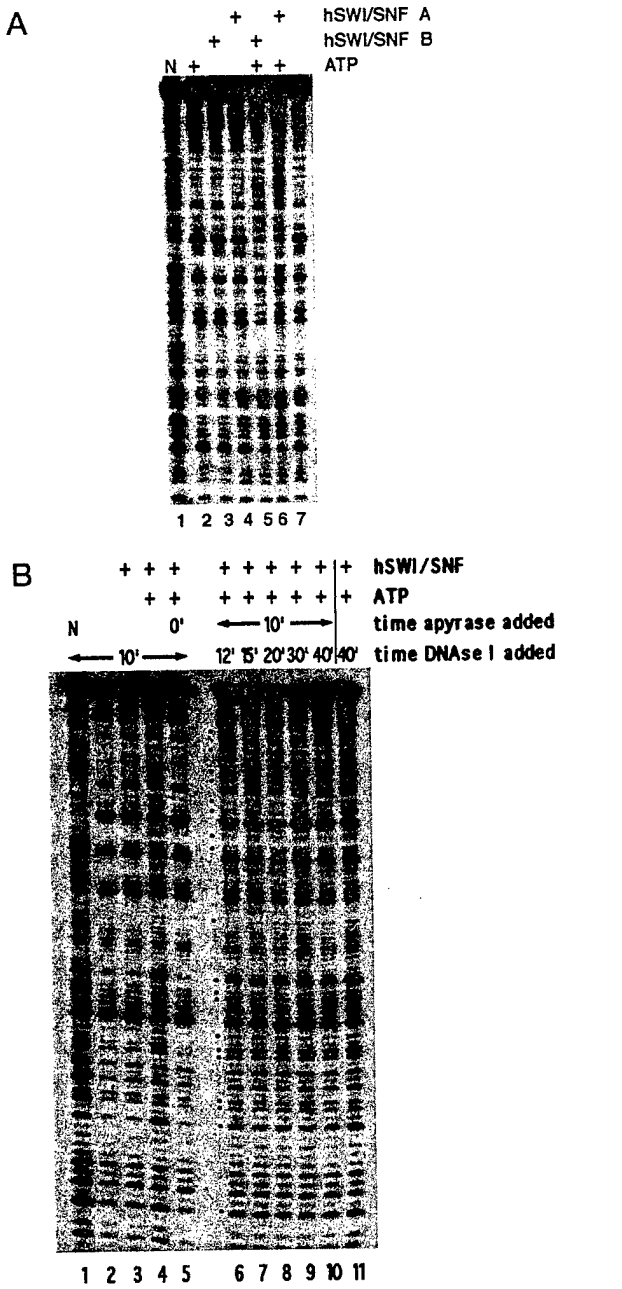

FIG. 2. *A*, nucleosome disruption by hSWI/SNF A and B is identical. Reaction conditions were as described for Fig. 1, except that, where indicated, reactions contained 4 mM ATP and 200 ng hSWI/SNF B fraction or 400 ng of hSWI/SNF A fraction. *B*, nucleosome disruption by hSWI/SNF is stable upon removal of ATP by apyrase. Reaction conditions were as described for Fig. 1, except that, where indicated, reactions contained 0.02 mM ATP and 300 ng of hSWI/SNF A fraction. Nucleosome disruption as assessed by DNase digestion 10 min after addition of hSWI/SNF is seen in *lane 4*; disruption after 40 min is seen in *lane 11*. Addition of 1 unit of apyrase prior to hSWI/SNF addition prevented nucleosome disruption (*lane 5*). A titration of apyrase concentration indicated that 0.1 unit was sufficient to prevent nucleosome disruption (data not shown). Apyrase was added to identical reactions 10 min after addition of hSWI/SNF (*lanes 6–10*), and disruption was assessed by DNase I digestion 2 min (*lane 6*), 5 min (*lane 7*), 10 min (*lane 8*), 20 min (*lane 9*), or 30 min (*lane 10*) after addition of apyrase. Reaction start times were staggered such that reactions presented in *lanes 6–11* were started 5 min after the reactions presented in *lanes 1–5*. *Solid dots* were placed at some of the bands or groups of bands where the frequency of DNase I cleavage was altered by hSWI/SNF. *N* represents naked DNA.

ATP Is Not Required to Maintain Disruption of Mononucleosomes—To characterize the alteration of nucleosome structure by hSWI/SNF, we sought to determine whether ATP was continuously required in order to maintain a disrupted pattern in the presence of hSWI/SNF. If ATP were continuously required for activity, that is, if the structural change induced by hSWI/SNF and ATP were transient, and the nucleosome reverted to its original form after ATP mediated disruption, then removal of ATP from the reaction prior to DNase I cleavage should generate the same 10-bp ladder of cleavage products seen when untreated nucleosomes are digested with DNase I. Alternatively, if the change in structure induced by hSWI/SNF is stable, then the altered DNase I digestion pattern should be maintained, even after ATP is removed from the reaction. To facilitate this experiment, the ATP concentration in the reactions was decreased to 20 μM, which is sufficient for disruption (Fig. 1, *lane 4*).

When apyrase, which cleaves ATP, was added to a reaction containing nucleosomes and ATP before the addition of hSWI/SNF, ATP-dependent nucleosome disruption was inhibited (Fig. 2*B*, compare *lanes 4* and *5*). In the reactions presented in *lanes 6–10*, identical samples containing nucleosomes, ATP, and hSWI/SNF were incubated for 10 min, were subsequently exposed to apyrase, and then were digested with DNase I at times ranging from 2 to 30 min following apyrase addition to determine whether the altered nucleosome would revert to its original structure. *Lanes 6–10* of Fig. 2*B* indicate that the altered DNase I digestion pattern was maintained for up to 30 min past the addition of apyrase. Other experiments indicate that the altered digestion pattern was maintained for up to 2.5 h after apyrase addition (data not shown). This result indicates that the alteration in nucleosome structure induced by hSWI/SNF is stable, even in the absence of ATP.

To confirm that hSWI/SNF could stably alter nucleosome structure, the nonhydrolyzable ATP analog, ATPγS, was used to competitively inhibit ATP hydrolysis by hSWI/SNF. Fig. 3 shows that concurrent addition of ATP and a 200-fold excess of ATPγS before addition of hSWI/SNF prevented nucleosome disruption for up to 60 min, presumably because ATPγS acts as a competitive inhibitor (*lanes 6* and *13*). When a 200-fold excess of ATPγS was added subsequent to hSWI/SNF addition, the altered DNase I digestion pattern was maintained (*lanes 7–11*), confirming that hSWI/SNF induced a stable change in nucleosome structure that was maintained in the absence of further hydrolysis. Addition of a 200-fold excess of AMP-PNP to the reaction did not inhibit hSWI/SNF activity (data not shown), probably reflecting a lower affinity of this analog for the ATP binding site.

ATP Is Not Required to Maintain Altered Supercoiling of Plasmid Templates—Previously, we demonstrated that the hSWI/SNF fractions can reduce the linking number of closed circular DNA that was assembled into a nucleosomal template (Kwon *et al.*, 1994). This result is consistent with the observation that hSWI/SNF fractions can increase the accessibility of mononucleosomes to nucleases such as DNase I and indicates that hSWI/SNF can alter chromatin structure on nucleosomal plasmid templates as well as on mononucleosome particles. In the experiment presented in Fig. 4*A*, ^{32}P-labeled, closed circular DNA was reconstituted into nucleosomes using octamers purified from HeLa cells and a *Xenopus* egg heat-treated extract that is competent for nucleosome assembly (Workman *et al.*, 1991b). The reconstituted template was purified by glycerol gradient centrifugation and was mixed with hSWI/SNF, ATP, and topoisomerase I. Following a 90-min incubation, the DNA was purified, and topoisomers were resolved by agarose gel electrophoresis. In an ATP-dependent manner, hSWI/SNF caused the appearance of a number of DNA topoisomers that have reduced mobility in the gel, indicating a loss of superhelical density (compare *lanes 2, 5,* and *7*). Addition of an 18-fold excess of ATPγS prior to the addition of hSWI/SNF prevented alteration of the template (*lane 6*). When an 18-fold excess of ATPγS was added to the reaction subsequent to addition of hSWI/SNF and ATP, DNA species with reduced superhelical density were still present (*lanes 9* and *10*), indicating that the structural alteration in the nucleosomal plasmid template caused by hSWI/SNF was stable, even upon inhibition by excess ATPγS. Similarly, addition of apyrase at the start of the reaction but prior to the addition of hSWI/SNF prevented alteration of the template (*lane 13*). When apyrase was added after the addition of hSWI/SNF and ATP, topoisomers with reduced superhelical density were present (*lanes 14–15*), indicating that removal of ATP from the reaction did not reverse the alteration in nucleosomal DNA structure. We therefore conclude that the alteration in structure of a nucleosomal plasmid template by hSWI/SNF is also maintained in the absence

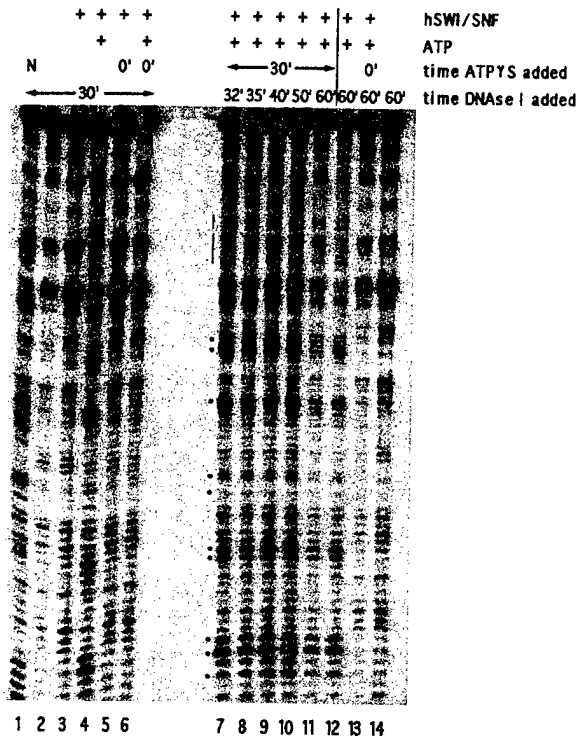

FIG. 3. **Nucleosome disruption by hSWI/SNF is stable when hSWI/SNF activity is competitively inhibited by excess ATPγS.** The experiment is similar to that presented in Fig. 2*B*. PH MLT nucleosomes were labeled at the *Bam*HI end. Where indicated, reactions contained 600 ng of hSWI/SNF B fraction, 0.02 mM ATP, and 4 mM ATPγS (200-fold excess). ATP-dependent nucleosome disruption 30 or 60 min after hSWI/SNF addition, as assayed by DNase I digestion, is seen in *lanes 4* and *12*. Substitution of excess ATPγS for ATP did not support nucleosome disruption (*lane 5* and Fig. 1). Addition of excess ATPγS to reactions containing ATP prior to addition of hSWI/SNF prevented nucleosome disruption when assayed 30 min (*lane 6*) or 60 min (*lane 13*) later. Excess ATPγS was added to identical reactions 30 min after addition of hSWI/SNF (*lanes 7–11*), and disruption was assessed by DNase I digestion 2 min (*lane 7*), 5 min (*lane 8*), 10 min (*lane 9*), 20 min (*lane 10*), or 30 min (*lane 11*) after addition of ATPγS. A time course of hSWI/SNF B activity indicated that the maximal change in the DNase I digestion pattern required 30 min (data not shown). Therefore, the reactions containing hSWI/SNF B, nucleosomes, and ATP were incubated for 30 min prior to ATPγS addition. This difference in time required for maximal disruption is not thought to represent a functional difference between the A and B fractions but instead reflects differences in hSWI/SNF concentration and the age of the preparations (data not shown). *Solid dots* and *lines* were placed at some of the bands or groups of bands where the frequency of DNase I cleavage was altered by hSWI/SNF. *N* represents naked DNA.

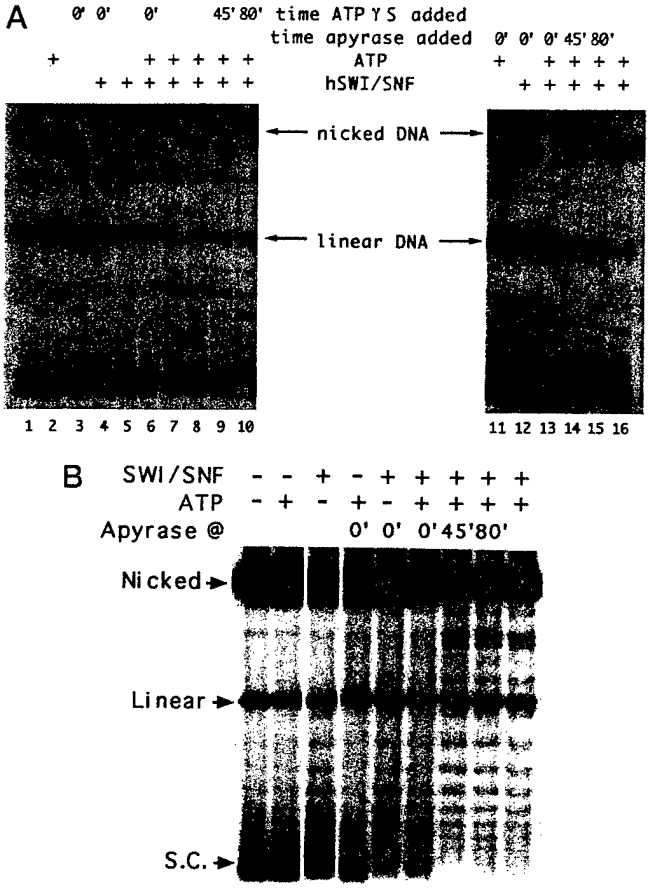

FIG. 4. **hSWI/SNF-mediated changes in supercoiling of nucleosomal plasmid templates are stable upon removal of ATP by apyrase or upon competitive inhibition by ATPγS.** *A*, reactions contained nucleosomal template (1 ng of DNA), 1 unit of topoisomerase I (Promega) and, where indicated, 360 ng of hSWI/SNF B fraction and 0.4 mM ATP. 7 mM ATPγS/$MgCl_2$ or 2.5 units of apyrase was added to the indicated reactions prior to hSWI/SNF addition (*lanes 3, 4, 6, 11, 12, 13*), 45 min after hSWI/SNF addition (*lanes 9, 14*), or 80 min after addition of hSWI/SNF (*lanes 10, 15*). Reactions were incubated for 90 min at 30 °C, except for the reactions presented in *lanes 8, 13,* and *16,* which were incubated for 45 min, and were stopped by addition of 6 μl of stop buffer (3% SDS, 100 mM EDTA, 50 mM Tris, pH 8.0, 25% glycerol, 2 μg/μl proteinase K). Reactions were then incubated at 37 °C for 90 min and resolved on 2% agarose, 50 mM Tris phosphate, pH 7.3, 1 mM EDTA gels for 40 h at 40 V. Gels were then dried and exposed to film. *B*, as in *A*, except that the gel was run in the presence of 0.5 μM chloroquine to resolve highly negatively supercoiled topoisomers. Reactions contained 225 ng of hSWI/SNF B fraction, 2 ng of chromatin template, and, where indicated, 1 unit of apyrase. In *lanes 5* and *6,* apyrase was added before hSWI/SNF. In *lane 9,* the reaction was stopped after 45 min. *S.C.* indicates supercoiled DNA.

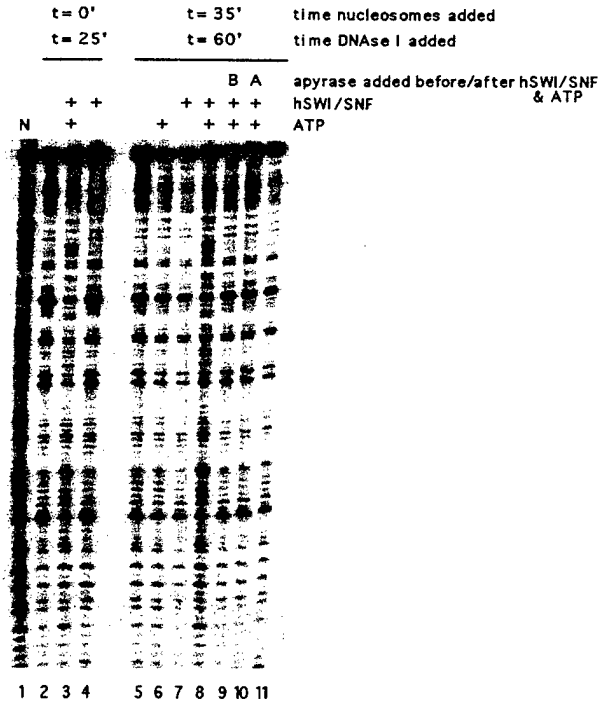

FIG. 5. **Nucleosome disruption by hSWI/SNF requires the simultaneous presence of nucleosomes and ATP.** Reactions were as described in Fig. 1. PH MLT nucleosomes were labeled at the *Eco*RI end. Where indicated, reactions contained 600 ng of hSWI/SNF B fraction and 0.02 mM ATP. In the control reactions (*lanes 2–4*), ATP-dependent nucleosome disruption is seen upon DNase I digestion 25 min after the start of the reactions (*lane 3*). For the reactions shown in *lanes 5–11,* reactions were initiated in the absence of nucleosomes. 1 unit of apyrase was added to the reaction prior to the addition of hSWI/SNF (*lane 9*) or 25 min after addition of hSWI/SNF (*lane 10*). PH MLT nucleosomes were added to each reaction (*lanes 5–11*) 35 min after the start. Nucleosome disruption was analyzed by DNase I digestion 60 min after the start of the reactions. No disruption was observed when apyrase was added after hSWI/SNF but before the nucleosomes (*lane 10*), indicating a concurrent requirement of ATP and nucleosomes for hSWI/SNF-mediated nucleosome disruption. *N* represents naked DNA.

of ATP hydrolysis.

To increase the resolution of the topoisomers showing a hSWI/SNF-induced reduction in supercoiling, similar reactions were resolved on agarose gels containing chloroquine (Fig. 4*B*). The change in superhelical density in the presence of hSWI/SNF and ATP is shown in *lane 9* (compare to *lane 1*). Addition of apyrase after the addition of hSWI/SNF did not change the distribution of topoisomers, again indicating that removal of ATP from the reaction did not reverse the alteration in nucleosomal DNA structure (*lanes 7–8*). The increase in resolution provided by the presence of chloroquine also indicated that hSWI/SNF has a small effect on nucleosome structure in the absence of ATP (*lanes 3, 5,* and *6*). ATP-independent effects of yeast SWI/SNF on DNA topology have previously been noted (Quinn *et al.*, 1996).

Estimations of the size of the hSWI/SNF complex (1 MD by gel filtration (Kwon *et al.*, 1994)) and of the purity of the fractions used (3–10%, as estimated by visual examination of silver-stained SDS-polyacrylamide gels, see "Materials and Methods") allowed a crude estimate of stoichiometry to be made in these experiments. We calculate that hSWI/SNF is approximately equimolar with mononucleosome particles at concentrations of hSWI/SNF where nucleosome disruption is maximal. For nucleosomal plasmid templates, we calculated that a ~20-fold excess of hSWI/SNF to template was sufficient to see maximal changes in supercoiling. This template contains 16 nucleosomes (on average), thereby resulting in an approximate equimolar ratio between hSWI/SNF and nucleosomes.

ATP Is Required to Initiate SWI/SNF Activity on Nucleosomes—The above results could be explained by a model in which ATP was necessary to produce an "active" version of hSWI/SNF, which would then be capable of altering nucleosome structure in the absence of further ATP hydrolysis. Alternatively, ATP hydrolysis could be required during hSWI/SNF disruption of the nucleosome. To distinguish between these possibilities, an order of addition experiment was performed in which hSWI/SNF was mixed with ATP, was subsequently treated with apyrase, and then was mixed with the nucleosomes (Fig. 5, *lane 10*). The results show that the unaltered 10-bp ladder of DNase I cleavage products was observed (compare *lanes 8* and *10*), indicating that nucleosome disruption required the simultaneous presence of hSWI/SNF, ATP, and nucleosomes. Since the ATPase activity of hSWI/SNF is

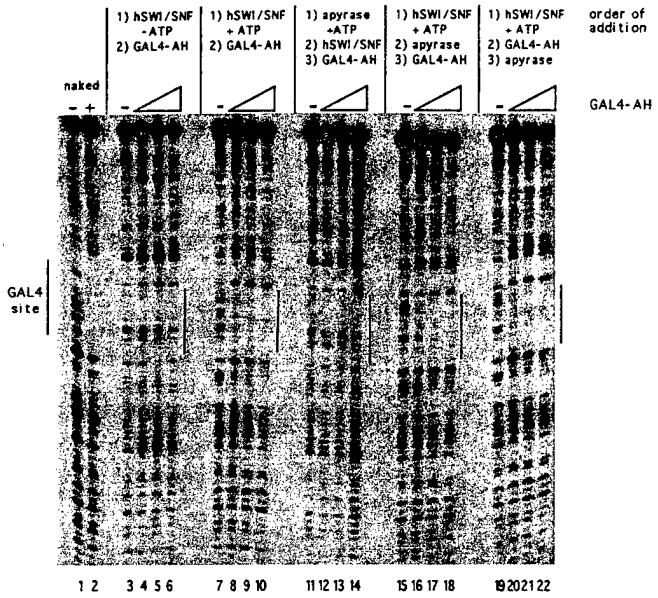

FIG. 6. **Facilitated binding of GAL4-AH to PH GAL4$_1$ mononucleosomes requires prior disruption by hSWI/SNF but does not require concurrent hSWI/SNF activity.** Reaction conditions were as described for Fig. 1. Where indicated, reactions contained 600 ng of hSWI/SNF B fraction, 4 mM ATP, and no GAL4-AH (*lanes 3, 7, 11, 15, 19*), 1×10^{-7} M GAL4-AH dimer (*lanes 4, 8, 12, 16, 20*), 1×10^{-6} M GAL4-AH dimer (*lanes 5, 9, 13, 17, 21*), or 1×10^{-5} M GAL4-AH dimer (*lanes 6, 10, 14, 18, 22*). In *lanes 3–10* reactions were treated with hSWI/SNF in the absence (*lanes 3–6*) or in the presence (*lanes 7–10*) of ATP for 30 min, and then GAL4-AH was added for another 30 min, and samples were assayed by DNase I digestion. If 1 unit of apyrase was added prior to hSWI/SNF addition (*lanes 11–14*), no facilitated GAL4-AH binding was subsequently observed. 1 unit of apyrase was added to the reactions presented in *lanes 15–18* 30 min after addition of hSWI/SNF; 10 min later, GAL4-AH was added for 30 min, followed by DNase I digestion. Reactions presented in *lanes 19–22* were identical to those in *lanes 15–18*, except that apyrase was added after the 30-min exposure to GAL4-AH, followed by DNase I digestion 10 min later. For comparison, naked PH GAL4$_1$ DNA (*lanes 1–2*) was incubated for 30 min at 30 °C, followed by an additional 30-min incubation in the absence (*lane 1*) or presence (*lane 2*) of 2×10^{-8} M GAL4-AH. Samples were then digested with DNase I. *Bars* span the sequences protected by GAL4-AH on naked and nucleosomal DNA.

stimulated by the presence of DNA (Kwon *et al.*, 1994), this experiment was repeated with 3–30 ng of free plasmid DNA present in the preincubation. As before, no alteration in the DNase I cleavage ladder was observed (data not shown). These results suggest that the hydrolysis of ATP is required for altering the structure of the nucleosome and not for modifying the hSWI/SNF. This experiment does not exclude the possibility that ATP modifies hSWI/SNF structure, but it indicates that even if such a modification occurs, it is not sufficient for nucleosome disruption.

Facilitation of GAL4 Binding Does Not Require Continued ATP Hydrolysis—Previous work has shown that if the nucleosome contains a transcription factor binding site, treatment of the nucleosome with hSWI/SNF and ATP facilitates the binding of transcription factors to it (*e.g.* GAL4 derivatives, TATA binding protein (Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a)). Addition of hSWI/SNF in the absence of ATP to nucleosomes containing a single GAL4 binding site at the dyad axis of symmetry results in no change in the DNase I digestion pattern, and, upon addition of increasing amounts of a GAL4 fusion protein, GAL4-AH, no binding to the GAL4 site was observed at the GAL4-AH concentrations utilized (Fig. 6, *lanes 3–6*). In contrast, when hSWI/SNF was added in the presence of ATP, a change in the DNase I digestion pattern was observed, and subsequent addition of increasing amounts of GAL4-AH resulted in specific occupancy of the GAL4 site (*lanes 7–10*), confirming previous observations. To determine whether the increased ability of a transcription factor to bind to an altered nucleosome requires continued ATP hydrolysis, nucleosomes were mixed with hSWI/SNF and ATP, and apyrase was added either before hSWI/SNF addition (*lanes 11–14*), after hSWI/SNF addition (*lanes 15–18*), or after the subsequent addition of GAL4-AH (*lanes 19–22*). Whereas addition of apyrase prior to the reaction prevented an increase in the ability of GAL4-AH to bind (*lanes 11–14*), addition of apyrase after addition of hSWI/SNF and ATP did not (*lanes 15–18*). Thus, prior alteration of nucleosome structure by hSWI/SNF is sufficient to allow an increase in GAL4-AH binding to nucleosomal DNA; concurrent ATP hydrolysis was not required.

hSWI/SNF Activity Is Not Due to Spontaneous Nucleosome Disruption—In the experiments presented here and in previous work, mononucleosome particles were present at a final concentration of 1.2×10^{-9} M, or approximately 0.12 ng/μl. It has recently been suggested that absolute concentration of nucleosomes may affect nucleosomal stability under certain reaction conditions; for example, when mononucleosomes are assembled from DNA containing *Xenopus* 5 S RNA sequences, they are not stable at 3 ng/μl under some conditions (Godde and Wolffe, 1995), while under other conditions they are stable at 0.3–0.6 ng/μl (Lee *et al.*, 1993). We therefore wanted to demonstrate that the nucleosomes were stable under the solution conditions used here.

We have previously reported DNase and micrococcal nuclease studies that demonstrate that nucleosomes assembled under the conditions reported here are stable (Imbalzano *et al.*, 1994a). To further support this conclusion, we performed a time course where free DNA or mononucleosome particles assembled from PH MLT DNA or PH MLT(+3) DNA, which has previously been shown to bind to yeast TBP and TFIIA when altered by hSWI/SNF, were incubated at the concentration and under reaction conditions used here and in previous work (1.2×10^{-9} M; 0.12 ng/μl), in the presence or absence of ATP (Fig. 7). At times ranging from 3 min to 2.5 h, identical reactions were loaded onto native 5%, 0.5 × TBE polyacrylamide gels and were subjected to electrophoresis at 6 V/cm. Since the nucleosome structure in the experiments presented was assayed at 10–70 min after the start of any given reaction, this experiment evaluates the state of the mononucleosome at times equal to and beyond the point where these and previous experiments were assayed. At times up to 90 min, no significant dissociation of nucleosomal DNA occurred, and the proportion of naked DNA present in any of the samples did not exceed 10%. A minor increase in the amount of free DNA did occur between 90 and 150 min, such that the percentage of free DNA after 2.5 h was between 10 and 15%. In no case was there rapid or significant spontaneous nucleosome disruption, and there was no effect of ATP on stability. We conclude that the mononucleosome particles are stable throughout and beyond the time parameters used in our experiments and are not subject to "spontaneous" nucleosome disruption. The alterations in nucleosome structure and the resulting increase in the ability of transcription factors to bind to nucleosomal DNA that we have observed are therefore due to the ATP-dependent changes caused by hSWI/SNF and the presence of the purified transcription factors.

DISCUSSION

At least two distinct nucleosome remodeling activities in eukaryotes, NURF and SWI/SNF, require ATP in order to alter nucleosome structure (Tsukiyama and Wu, 1995; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a; Cote *et al.*, 1994). The data presented here distinguish between possible mechanisms of

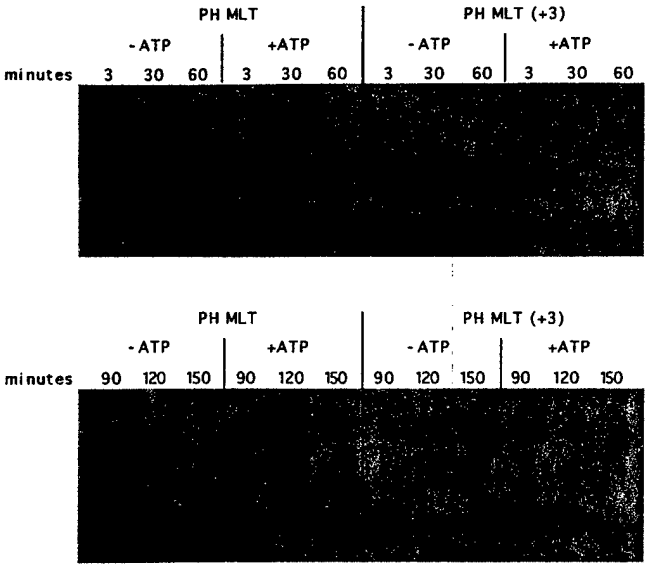

FIG. 7. **Mononucleosome particles are stable under the reaction conditions and at the nucleosome concentrations utilized for up to 2.5 h after the reaction start time.** Nucleosomes were assembled from PH MLT or PH MLT(+3) DNA. Identical reactions (conditions were as described in Fig. 1) containing naked or nucleosomal DNA were incubated at 30 °C. Where indicated, reactions contained 4 mM ATP. At the indicated times, one naked DNA and one nucleosomal DNA reaction were removed from the 30 °C bath and loaded directly onto adjacent lanes of a native 5%, 0.5 × TBE polyacrylamide gel. A second gel was used to assay nucleosomes at 90–150 min after the start of the reactions. Electrophoresis was performed at 6 V/cm. Gels were then dried and exposed to film or a phosphorimager screen (Molecular Dynamics). This experiment was performed with nucleosome particles that had been assembled 24 days previously. Thus, storage of the nucleosome particles for 24 days at 4 °C had no effect on nucleosome stability, and, in fact, we have observed that assembled nucleosomes are stable for up to 6 weeks post-assembly (data not shown). These observations are in agreement with the findings of Li and Wrange (Li and Wrange, 1993; Li and Wrange, 1995).

action by hSWI/SNF and indicate that ATP hydrolysis is not required for an activity that continually weakens histone-DNA contacts on the surface of a nucleosome. Instead, hSWI/SNF is able to alter nucleosome structure in a manner that remains completely stable after removal of ATP or after competitive inhibition of ATP hydrolysis. We do not know whether hSWI/SNF presence is required to maintain the disrupted structure. It is possible that hSWI/SNF has an ATP-dependent activity that is necessary to alter nucleosome structure and a separate activity (not ATP-dependent) that is necessary to maintain the altered structure. Recent work has demonstrated that the yeast SWI/SNF complex both binds to and can alter the topology of naked DNA in an ATP-independent manner (Quinn *et al.*, 1996). The relevance of these observations to nucleosome disruption and whether hSWI/SNF possesses similar properties remain unclear. However, the fact that hSWI/SNF can cause a small ATP-independent reduction in supercoiling on a plasmid nucleosomal template (Fig. 4*B*) may suggest that there is an ATP-independent association of hSWI/SNF with the template. Alternatively, hSWI/SNF might introduce a stable change in nucleosome structure that is maintained in the complete absence of hSWI/SNF activity.

It is possible that hSWI/SNF removes histones from the nucleosome in an ATP-dependent manner, and this altered structure is maintained because histones will not reassemble into a nucleosome spontaneously at low salt concentrations. It has not been possible in this or other systems to determine directly the fate of the histones after disruption; however, several results indicate that the histone octamer has not been removed. The DNase I digestion pattern following disruption of mononucleosomes by hSWI/SNF is not identical to that of naked DNA (this report; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994a). This does not appear to be the result of a mixed population of nucleosomal and naked DNA, as GAL4 binding can be facilitated by hSWI/SNF on all templates in a given reaction, as demonstrated by essentially complete protection of the binding site (Fig. 6). In addition, previous results demonstrated that hSWI/SNF could facilitate TBP binding in a manner that still required appropriate rotational positioning of the TATA box on the nucleosome surface, indicating that histones are likely to still be present (Imbalzano *et al.*, 1994a). Finally, we have been unable to visualize free DNA following hSWI/SNF activity by gel shift analysis, even after adding excess nonspecific DNA or histone binding proteins to act as an acceptor for histone transfer (data not shown). There is also no evidence that hSWI/SNF modifies any of the core histone proteins; histones are not phosphorylated by hSWI/SNF nor are they modified sufficiently to alter their migration on SDS-polyacrylamide or Triton/acid/urea gels (data not shown). This latter finding strongly suggests that the N-terminal tails of each of the core histones are neither removed nor acetylated.

The spectrum of nucleotides that will function with hSWI/SNF in disruption is essentially identical to that of *Drosophila* NURF (Tsukiyama and Wu, 1995), which also disrupts nucleosome structure in an ATP-dependent manner. It is believed that the Brg1 and/or hBrm protein encodes the ATPase of hSWI/SNF (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994) and that ISWI, a *Drosophila* protein with similarity to SWI2/SNF2 (Elfring *et al.*, 1994), encodes the ATPase domain of NURF (Tsukiyama *et al.*, 1995). These genes all display extensive homology in their ATPase domains, consistent with their similar nucleotide requirements.

We have used three separate protocols to assess the effect of ATP depletion on nucleosome disruption: alteration of DNase I digestion of a mononucleosome, alteration of supercoiling in a circular plasmid, and facilitation of GAL4 binding to nucleosomal DNA. In all three cases the effects of hSWI/SNF were fully maintained in the absence of ATP. These data are consistent with the hypothesis that all three effects are a consequence of the same underlying change in nucleosome structure induced by hSWI/SNF.

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Activator-dependent regulation of transcriptional pausing on nucleosomal templates

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Promoter–proximal pausing during transcriptional elongation is an important way of regulating many diverse genes, including human c-*myc* and c-*fos*, some HIV genes, and the *Drosophila* heat shock loci. To characterize the mechanisms that regulate pausing, we have established an in vitro system using the human *hsp70* gene. We demonstrate that nucleosome formation increases by >100-fold the duration of a transcriptional pause on the human *hsp70* gene in vitro at the same location as pausing is observed in vivo. Readthrough of this pause is increased by an activator that contains the human heat shock factor 1 (HSF1) transcriptional activation domains. Maximal effect of the activator requires that the system be supplemented with fractions that have hSWI/SNF activity, which has been shown previously to alter nucleosome structure. No significant readthrough is observed in the absence of activator, and neither the activator nor the hSWI/SNF fraction affected elongation on naked DNA; therefore, these results suggest that an activator can cause increased readthrough of promoter-proximal pausing by decreasing the inhibitory effect of nucleosomes on transcriptional elongation.

[*Key Words:* Transcriptional pausing; nucleosomes; *hsp70*; heat shock factor 1; hSW1/SNF complex; transcriptional activators]

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Mechanisms of gene regulation at the level of transcriptional elongation have been well-characterized in prokaryotes. The identification of eukaryotic counterparts to these mechanisms is just beginning. Examples of regulated elongation in mammalian cells include the *myc, myb, fos, mos,* and *ada* genes, which exhibit a regulatory block to transcription near their 5′ ends (for review, see Spencer and Groudine 1990). The *Drosophila hsp70* gene is also regulated at the level of elongation (Gilmour and Lis 1986; Rougvie and Lis 1988; Giardina et al. 1993). A paused, transcriptionally engaged RNA polymerase ternary complex has been found over a relatively narrow promoter–proximal region on *hsp70* (Rasmussen and Lis 1993). In response to heat shock, not only does the rate of initiation increase but the transit time of polymerase through the pause is drastically reduced. Similar pauses have been found in *Drosophila* on other heat shock genes (e.g., *hsp26*), metabolic genes (*gadph-1* and *gadph-2*), and structural genes (β1-*tubulin*), so this phenomenon may be relatively widespread (Rougvie and Lis 1990).

What causes and what releases a eukaryotic pause remains mostly a mystery. Pausing on HIV-1 is at least partially determined by an RNA-encoded TAR element and partially relieved by the Tat *trans*-activator (for review, see Cullen 1990). On human c-*myc* (Krumm et al. 1995) and *Drosophila hsp70* (Lee et al. 1992) no such discrete elements have been found. Nonetheless, in vitro transcription studies on chromatin templates have shown that nucleosomes greatly enhance sequence-specific pausing (Izban and Luse 1991). This enhanced pausing might be a consequence of the need for nucleosomes to be displaced during the transcription process (Clark and Felsenfeld 1992; Studitsky et al. 1994). Hence, it is plausible that nucleosomes might play a role in the control of eukaryotic transcriptional elongation by causing specific, regulatable pauses. These pauses might then be regulated by transcriptional activators, by elongation factors like TFIIF (Flores et al. 1989) and TFIIS (Reinberg and Roeder 1987; Reines et al. 1989), or by chromatin-reorganizing factors like the SWI/SNF complex (Winston and Carlson 1992; Cairns et al. 1994; Cote et al. 1994; Imbalzano et al. 1994; Kwon et al. 1994) or the *Drosophila* NURF complex (Tsukiyama and Wu 1995).

Activators have been implicated primarily in regulating transcriptional initiation on both naked and nucleosomal DNA, and current evidence suggests that they achieve their effects in several different ways. Direct contacts between activators and components of the gen-

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eral transcription machinery have been proposed to regulate pre-initiation complex formation and DNA melting at the promoter (for review, see Ptashne and Gann 1990; Kingston and Green 1994; Tjian and Maniatis 1994). Studies on chromatin templates show that activators can relieve nucleosomal inhibition of transcription, possibly through contacts with the general transcription machinery and with complexes like SWI/SNF that directly destabilize chromatin structure (for review, see Workman and Buchman 1993). Finally, activators may also play a direct role in the regulation of elongation. An in vivo study by Yankulov et al. (1994) demonstrated that a variety of activators are able to stimulate elongation through pausing and termination sites on stably transfected reporter constructs, and Krumm et al. (1995) found that enhancers can increase readthrough of promoter–proximal pausing.

This paper provides evidence that the human heat shock factor 1 (HSF1) transcriptional activation domains can regulate elongation through the human *hsp70* promoter in vitro and suggests that this is accomplished by overcoming a nucleosome-dependent block to transcriptional elongation. Specifically, we show that transcriptional pausing on *hsp70* is increased dramatically by the presence of nucleosomes. Pausing is released when reactions contain the HSF1 activation domain as part of a GAL4 fusion protein, and maximal release also requires fractions with hSWI/SNF activity. Neither GAL4–HSF nor the hSWI/SNF fraction affect elongation on naked templates, suggesting that their effects on elongation are specific to the nucleosome-dependent block.

Results

A transcriptional pause is centered at +45 on the human hsp70 *gene in vivo*

Previous work has identified a regulated promoter–proximal transcriptional pause at several mammalian and *Drosophila* loci (for review, see Spencer and Groudine 1990). In particular, on the *Drosophila hsp70* gene, paused polymerase molecules have been mapped in vivo by a variety of methods to between +20 and +30 relative to the start of transcription (Gilmour and Lis 1986; Rougvie and Lis 1988; Giardina et al. 1992; Rasmussen and Lis 1993). To understand how regulation of pausing might be achieved, we first determined whether the phenomenon of *hsp70* promoter–proximal pausing is conserved in humans (a result expected from the extraordinary conservation of the proteins involved among metazoans) and then established the human *hsp70* promoter as an in vitro system to examine the control of pausing. In this way we were able to compare pausing in our human cell-free system to pausing at the same locus in human cells.

To examine transcriptional pausing on the *hsp70* gene in vivo, potassium permanganate was used to footprint RNA polymerase open transcription complexes in HeLa cells. Permanganate freely diffuses through cell membranes and modifies thymine residues of single-stranded regions of DNA; hence, it is particularly useful for detecting regions of DNA in vivo that have been pulled apart by a paused, transcribing RNA polymerase molecule (Sasse-Dwight and Gralla 1989; Kainz and Roberts 1992; Wang et al. 1992). Modified bases can subsequently be changed to nicks by piperidine cleavage, and cleavage products can be examined by ligation-mediated PCR (LMPCR) (Mueller and Wold 1989). This permanganate footprinting protocol has been used previously to detect transcriptional pausing on the human c-*myc* and *Drosophila hsp70* genes (Giardina et al. 1992; Krumm et al. 1992).

When growing HeLa cells were treated with permanganate, subsequent analysis of the cleavage products revealed a locus of coding-strand hypersensitivity centered at +45 compared with DNA treated with permanganate in vitro (Fig. 1A, lanes 1,2), which would not contain melted regions caused by RNA polymerase molecules. In contrast, there were only two minor hypersensitive sites at +30 and +48 on the noncoding strand (Fig. 1A, lanes 3,4). Such strand specificity is expected for footprints of transcriptionally engaged RNA polymerase molecules, because permanganate access to the noncoding strand is inhibited by the nascent transcript or by tight binding to RNA polymerase itself. Further evidence that the observed hypersensitivities were transcription-related was provided by permanganate footprinting studies done in the presence of the transcriptional inhibitors α-amanitin or actinomycin-D. When HeLa cells were treated with either of these reagents prior to permanganate footprinting, the +45 hypersensitivity was reduced (Fig. 1B, lanes 3–6). Hence, it is likely that there is a paused, transcriptionally engaged RNA polymerase molecule at approximately +45 on the human *hsp70* gene in vivo.

When human cells are heat shocked, *hsp70* transcription increases ~20-fold (Morimoto 1993). Nonetheless, when heat-shocked HeLa cells were subjected to permanganate analysis, permanganate hypersensitivity in the human *hsp70* gene was similar to that seen in normally growing cells (Fig. 1C, lanes 1,2). This observation matches similar findings about the *Drosophila hsp70* gene (Giardina et al. 1992) and implies that RNA polymerase still pauses at this sequence following activation of the promoter; however, the pause must be less penetrant or shorter in duration to account for the increase in full-length transcript.

Nucleosome-dependent pausing is observed on hsp70 *in vitro*

No long pauses are observed when naked *hsp70* DNA is transcribed by RNA polymerase II in vitro (see below). Because Izban and Luse (1991) had shown that nucleosomes can enhance sequence-specific pausing, we examined whether nucleosomes cause RNA polymerase II to pause at specific positions on the human *hsp70* promoter. We used a modification of the protocol of Izban and Luse to examine elongation of RNA polymerase at high nucleotide concentrations on nucleosomal templates. First, four point mutations were made in a tem-

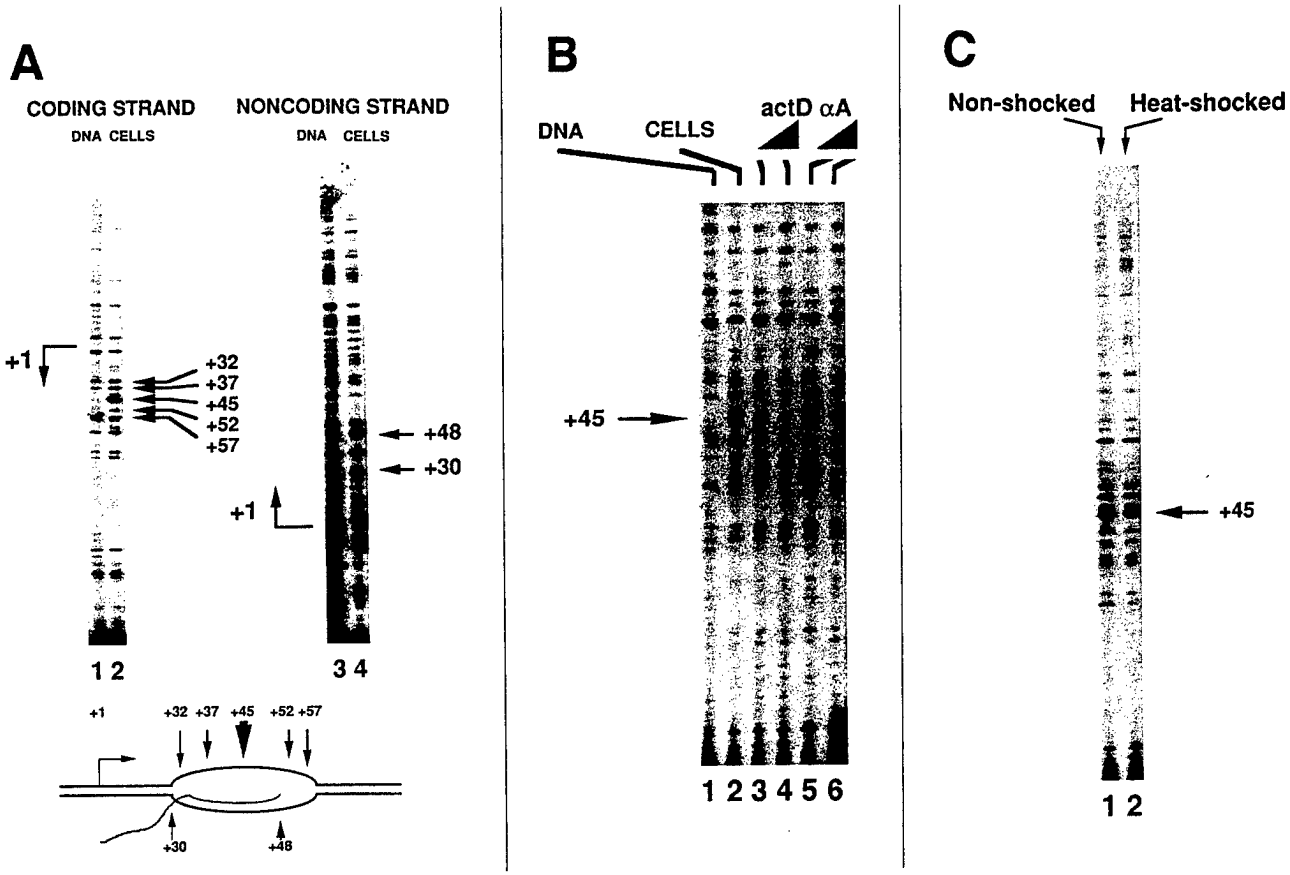

Figure 1. Permanganate hypersensitivity on the human *hsp70* gene in vivo. (*A*) Intact HeLa cells (lanes *2,4*) or genomic DNA (lanes *1,3*) were treated with potassium permanganate and coding strand cleavages (lanes *1,2*) or noncoding strand cleavages (lanes *3,4*) were visualized by LMPCR. (*B*) HeLa cells were not treated (lane *2*), or treated for 2 hr with 1 μg/ml (lane *3*) or 5 μg/ml (lane *4*) of actinomycin D, or 1 μg/ml (lane *5*) or 5 μg/ml (lane *6*) of α-amanitin, followed by potassium permanganate treatment and LMPCR to visualize coding-strand cleavages; genomic DNA (lane *1*) is shown for comparison. (*C*) HeLa cells were heat-shocked at 43°C for 1 hr (lane *2*) or not heat-shocked (lane *1*), and coding strand sensitivity to permanganate was visualized with LMPCR.

plate containing the natural human *hsp70* promoter so that there were no guanosine residues in the first 15 bases of the transcript. This modified *hsp70* template was tethered to polystyrene beads to facilitate changes of nucleotide mixes during transcription (Arias and Dynan 1989). Transcription was then initiated using HeLa basic transcription factors (partially purified to remove contaminating nucleotides), labeled UTP, and low concentrations of ATP and CTP. Under these "G-less" conditions, templates were generated that contain RNA polymerase artificially stalled at +15 with a labeled nascent transcript. Such prestalling of RNA polymerase complexes prior to nucleosome assembly was necessary to avoid the inhibitory effects of nucleosomes upon initiation (Knezetic and Luse 1986; Lorch et al. 1987; Matsui 1987), so that we could specifically examine the effects of nucleosomes upon elongation.

After nucleotides and loosely bound proteins were washed away from the prestalled RNA polymerase II complexes with 1% Sarkosyl, nucleosomes or other factors were added as desired and transcription was permitted to continue by adding back high concentrations of all four unlabeled nucleotides. By performing a time course following the addition of nucleotides, the extent of pausing at specific positions in the *hsp70* gene could be determined directly by visualizing the end-labeled transcripts.

On naked *hsp70* DNA transcribed by this protocol, there was no evidence of a long-lasting specific pause (Fig. 2, lanes 1–3), but if the template was assembled into nucleosomes prior to the addition of nucleotides, RNA polymerase elongation was dramatically inhibited and almost all RNA polymerase molecules remained stopped at or before +46 to +49 (Fig. 2, lanes 4–6), the location of pausing in vivo. Specifically, the major pause sites that we observed were at +46 to +49 (20%–50% of total counts over multiple experiments) and at +27 to +30 (25%–50%). Quantitation of the latter pause is difficult because of its proximity to the dye front, and its significance is not currently known, as no pausing at this location was observed in vivo. Both of these pauses are stable for >6 hr, the longest time point tested (data not shown).

The short transcripts that we observed could have

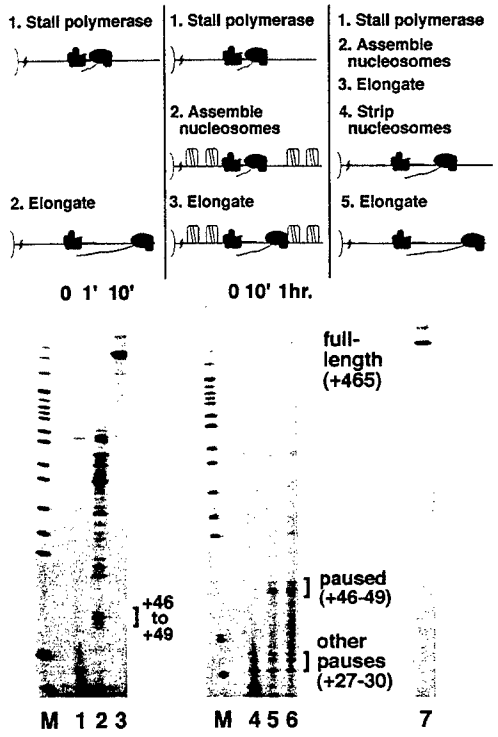

Figure 2. Transcriptional pausing on the human *hsp70* gene in vitro. Transcription complexes were stalled at +15 on human *hsp70* template pSAB8 (containing a short G-less region from +1 to +15), washed, and either elongated immediately in the presence of all four unlabeled nucleotides (lanes *1–3*) assembled into nucleosomes and then elongated (lanes *4–6*), or assembled and elongated as in lane *6*, and then stripped of nucleosomes with a Sarkosyl wash and elongated again (lane *7*); the period of elongation for each reaction is specified above lanes *1–6*, and was 1 hr for lane *7*.

been caused either by transcription termination or by pausing. To distinguish between these possibilities, nucleosomes were removed from reactions displaying the putative paused transcripts (e.g., lane 6) by washing with 1% Sarkosyl. Transcription was then permitted to continue by adding back all four nucleotides. At the end of this protocol, only full-length transcripts were observed (lane 7); hence, the paused transcripts in Figure 2 were the result of stably paused RNA polymerase that could elongate after removal of the nucleosomes and were not caused by premature termination.

Promoter–proximal areas of the hsp70 *transcribed region are nucleosomal in vitro and in vivo*

To further support this nucleosome-dependent model for *hsp70* pausing, we next addressed whether templates that contained the paused polymerase were actually assembled into nucleosomes. Nucleosomal DNA is refractory to cleavage by restriction enzymes such as *Bam*HI, so if nucleosomes were required for the pause at +46 to +49, then these templates should not contain an accessible *Bam*HI site at +150. When stalled artificially, unassembled *hsp70* templates were cleaved with *Bam*HI, the templates were cut at +150, so subsequent elongation yielded nearly all short transcripts (Fig. 3A, lane 1). This control confirmed the accessibility of the *Bam*HI restriction site in the absence of nucleosomes. When the templates were assembled into nucleosomes and then treated with *Bam*HI, almost all transcripts were paused at or before +46 to +49, as discussed above (Fig. 3A, lane 2). A small fraction (10%) reached the *Bam*HI site at +150, suggesting that they did not pause because they were unassembled. If these assembled and digested reactions were stripped of nucleosomes, all paused transcripts elongated to full-length transcripts (lane 3), whereas the backround of short, unassembled transcripts remained constant at 10%. Therefore, all paused transcripts must have arisen from assembled templates. [This procedure was adapted from that described by Morse (1989).] The same experiment was conducted using restriction enzymes that cleave at various points along the template with similar results (Fig. 3B). Assembled templates were resistant to cleavage and gave full-length transcripts upon removal of the nucleosomes (Fig. 3B, lanes 1,3,5,7). Unassembled templates were cut to give almost exclusively short transcripts (Fig. 3B, lanes 2,4,8), with the exception of those treated with *Pst*I, which also cleaves upstream of the start site thereby eliminating the transcript (Fig 3B, lane 6). From these experiments, it was concluded that all transcribed templates contain a nucleosome downstream of the start site.

We next examined whether the corresponding region of the *hsp70* gene is nucleosomal in vivo. When DNA from HeLa nuclei treated with micrococcal nuclease was transferred to nylon membrane and probed with a fragment of the *hsp70* gene stretching from +150 to +235, a ladder of bands with the characteristic spacing of nucleosomes was observed (Fig. 3C). This pattern was not observed on naked DNA, and no signal was observed on an identical blot of DNA from micrococcal nuclease-treated mouse nuclei (data not shown), implying that the observed pattern is not an artifact either of the intrinsic susceptibility of *hsp70* DNA to micrococcal nuclease cleavage or of hybridization specificity. We have not detected specific nucleosome positioning in this region as measured by indirect end-labeling (data not shown).

The pause observed on hsp70 *in vitro is promoter-dependent*

To demonstrate that the pattern of pausing that we measured in vitro was specific to the *hsp70* gene, we tested the adenovirus major late promoter in a similar in vitro assay. When this promoter instead of the *hsp70* promoter was transcribed with the protocol of Figure 2, inhibition of elongation and enhancement of sequence-specific pausing was observed (Izban and Luse 1991; Fig. 4A, lane 5), but there was no locus of sharply defined pausing at +46 to +49 like that on the *hsp70* gene (Fig. 4A, lane 1).

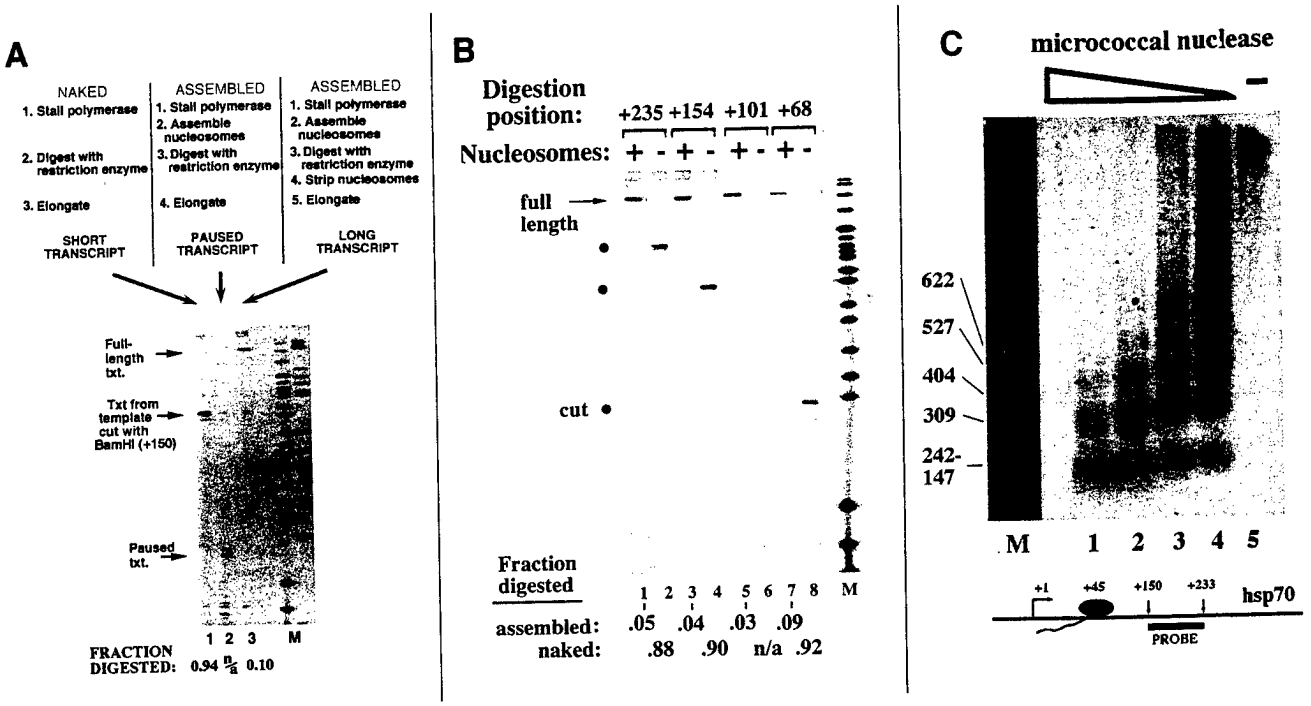

Figure 3. Nuclease accessibility of the *hsp70* gene in vitro and in vivo. (*A*) Naked templates with artificially stalled transcription complexes were either digested with *Bam*HI and elongated (lane *1*), assembled into nucleosomes and then digested with *Bam*HI and elongated (lane *2*), or assembled, digested, stripped of nucleosomes, and then elongated (lane *3*). The ratio of short transcripts to full-length ones is quantitated underneath appropriate lanes. (*B*) Restriction enzyme accessibility tests identical to the ones in *A* were performed with *Sac*II (lanes *1,2*), *Bam*HI (lanes *3,4*). *Pst*I (lanes *5,6*), and *Sty*I (lanes *7,8*). Lanes *2*, *4*, *6*, and *8* were digested as naked templates. Lanes *1*, *3*, *5*, and *7* were digested after nucleosome assembly. Underneath each lane, the fraction of transcripts cut by restriction enzyme is indicated. (*C*) DNA from HeLa nuclei treated with 0 (lane *5*), 30 (lane *4*), 90 (lane *3*), 270 (lane *2*), or 810 (lane *1*) units of micrococcal nuclease was Southern blotted and probed with a fragment of *hsp70* DNA from +150 to +235. The marker lane contains pBR322 DNA digested with *Msp*I.

Hence, specific pausing at +46 to +49 was unique to the *hsp70* gene.

Next, we carried out a variety of controls to address the possibility that the pause observed was of merely fortuitous length. We were concerned that the length of the linear template used in the protocol would cause nucleosomes to become positioned in a manner that would determine the location of the pause site. We found, however, that the pausing observed on *hsp70* in vitro was independent of template length: The same +46 to +49 pause was observed on a mixture of templates containing from 335 to 395 bp of downstream sequence (Fig. 4A, lane 3). Next, the independence of +46 to +49 pausing from the location of initial artificial stalling at +15 was demonstrated by examining pausing while varying the location of the artificial stall. We obtained the same results if RNA polymerase was stalled at +1 prior to nucleosome assembly (by initially withholding nucleotides entirely), as we did if we permitted RNA polymerase to progress to +15 prior to assembly (by adding three nucleotides) (Fig. 4B). Taken together, these experiments show that the specific nucleosome-dependent pausing that we observe on *hsp70* in vitro arises from some quality intrinsic to the *hsp70* gene rather than from some aspect of our in vitro system.

Activator-dependent release of pausing

Activation of the human *hsp70* gene following heat shock is caused by the binding of an activated form of human HSF1 to the heat shock element. The transcriptional activation domains of human HSF1, amino acids 202–529 (Green et al. 1995; Zuo et al. 1995), were therefore tested for their ability to relieve pausing on the *hsp70* gene in vitro. We used a GAL4 DNA binding domain to tether the HSF1 activation domains to the promoter region because the magnesium and detergent requirements for DNA binding by intact HSF1 purified from human cells (Schuetz et al. 1991) are incompatible with the experimental protocol used here.

When GAL4–HSF was prebound to an *hsp70* promoter containing five GAL4 DNA-binding sites, there was some increased readthrough of the +46 to +49 pause (Fig. 5A, cf. lanes 1 and 2). Because GAL4–HSF addition alone did not promote a large amount of readthrough, a search for accessory factors was undertaken. One possible class of accessory factors are elongation factors known to relieve pausing in other in vitro systems, such as TFIIF (Flores et al. 1989) and TFIIS (Reinberg and Roeder 1987; Reines et al. 1989). Alone or in the presence of GAL4–HSF, though, these two factors had no

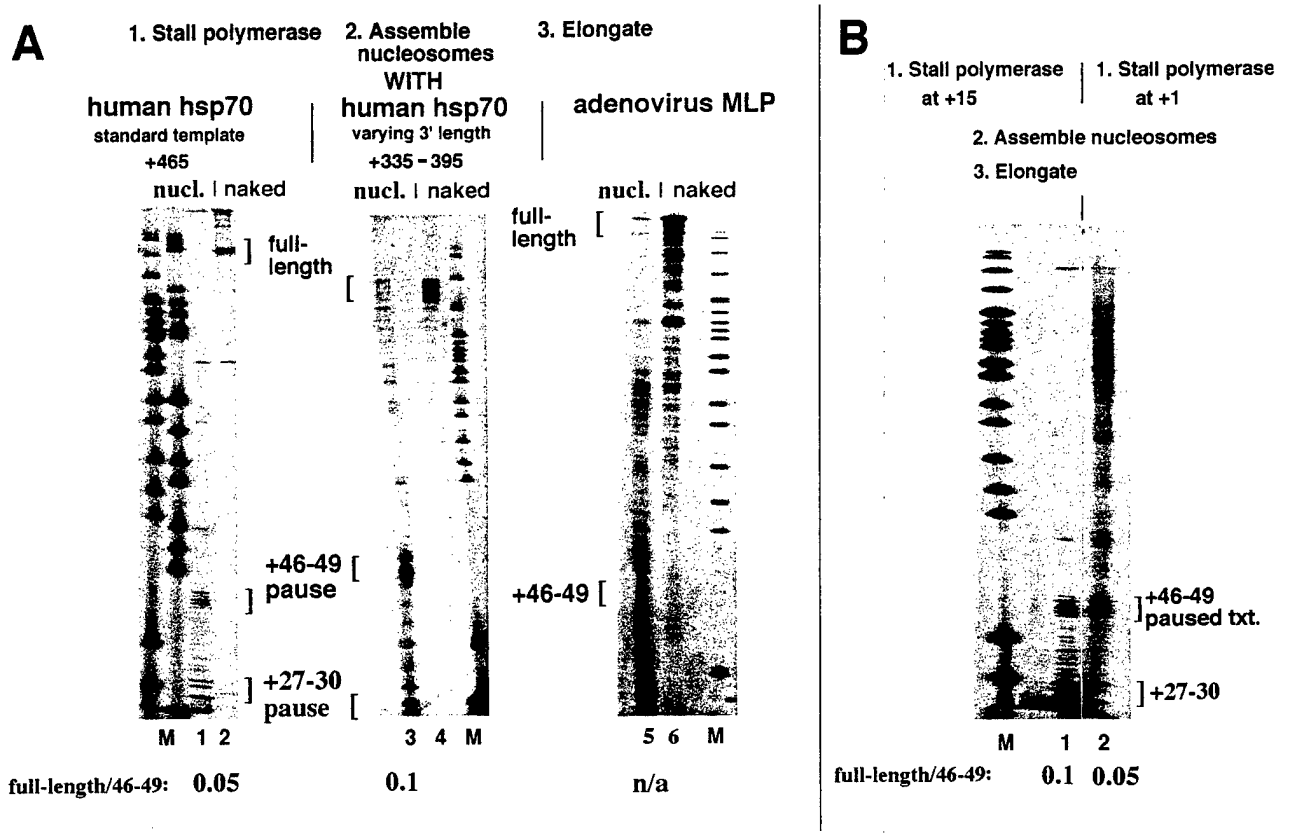

Figure 4. The in vitro pause is promoter-dependent but independent from template length and the location of initial artificial stalling. (*A*) Pausing of RNA polymerase on various templates: pSAB8, human *hsp70* to +465, nucleosomal (lane *1*) or naked (lane *2*); pSABmix, human *hsp70* to +335, +355, +375, +395, nucleosomal (lane *3*) or naked (lane *4*); pML5–4NR, adenvirus major late promoter (MLP) with 637 bp downstream sequence, nucleosomal (lane *5*) or naked (lane *6*). In all cases, elongation was permitted to occur for 1 hr. The ratio of full-length transcript to +46 to +49 paused transcripts is listed for nucleosomal DNA below each relevant lane. The ratio of +46 to +49 transcript to +27 to +30 transcript was 0.9 in lane *1* and 0.3 in lane 3. (*B*) Transcription complexes were initially stalled at +1 by withholding all nucleotides (lane *2*) or at +15 by supplying three nucleotides (lane *1*), followed by subsequent nucleosome assembly and elongation in the presence of all four nucleotides. The ratio of full-length transcripts to +46 to +49 transcripts is listed below each lane. The ratio of +46 to +49 to +27 to +30 transcripts was 0.5 in lane *1* and 0.9 in lane *2*. Because transcription complexes stalled at +1 were stalled as preinitiation complexes rather than elongation complexes, the Sarkosyl washing step prior to nucleosome assembly, which would strip these preinitiation complexes, had to be omitted from the standard protocol. The lack of Sarkosyl washing accounts for the increased background in lane *2* and in other unwashed reactions containing elongation complexes (data not shown).

effect upon the exent of readthrough of pausing (data not shown).

Because the pause at +46 to +49 was nucleosome dependent, we next investigated whether fractions containing hSWI/SNF activity could effect release of the pause. The yeast SWI/SNF complex has been implicated by genetic and biochemical studies to be involved in chromatin reorganization and gene activation (for review, see Winston and Carlson 1992), and the purified complex has been shown to possess an ATP-dependent nucleosome-disrupting activity (Cote et al. 1994). Fractions enriched for homologous complexes from HeLa cells display similar ATP-dependent reorganizing abilities (Imbalzano et al. 1994; Kwon et al. 1994). We therefore tested fractions that contain human SWI/SNF activity to see whether they would enhance relief of pausing on the *hsp70* gene. Readthrough was greatly enhanced by the presence of either the human SWI/SNF A fraction (Fig. 5A, lane 4) or the SWI/SNF B fraction (data not shown), two highly enriched, chromatographically distinct fractions that both contain human homologs of the yeast SWI2/SNF2 protein (Imbalzano et al. 1994; Kwon et al. 1994). At the same time, the amount of paused transcript decreased correspondingly (see legend to Fig. 5). These fractions had no effect upon readthrough when an ATP analog with a nonhydrolyzable γ phosphate was employed during the reaction (ATPγS, data not shown). Interestingly, the same fractions also had no effect on pausing in the absence of activator (Fig. 5A, lane 3; see below). Hence, the actions of both an activator and an ATP-dependent activity were required for maximal relief of nucleosome-dependent pausing.

Because readthrough of pausing required the GAL4–HSF protein regardless of any accessory factors present,

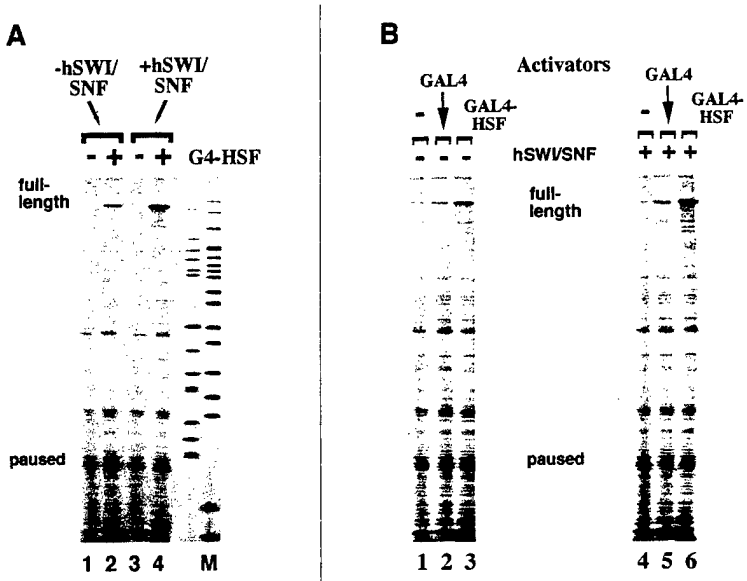

Figure 5. Effects of HSF1 activation domain and the hSWI/SNF fraction upon pausing. (*A*) Template pSAB12 (a *hsp70* transcription template similar to the one used in Figures 2–3, but containing 5 GAL4 DNA-binding sites) was subjected to the standard elongation protocol of artificially stalling RNA polymerase complexes at +15, assembling nucleosomes, and continuing elongation. (Lanes *3,4*) 1 unit of hSWI/SNF preincubated in the presence of 4 mM ATP 15 min prior to the final elongation step of the reactions. (Lanes *2,4*) A 1.2-fold molar excess of GAL4–HSF over binding sites preincubated with naked template for 15 min, and then present during all steps of the transcription. (Lane *1*) Neither activator nor hSWI/SNF. Between lanes *2* and *4*, the +46 to +49 transcript decreases from 183 to 153 units, and smaller transcripts decrease from 292 units to 242 units, for a total decrease of 80 units. At the same time, the full-length transcripts increase from 32 units to 107 units, for a total increase of 75 units. The units here are arbitrary light units as quantified by a Molecular Dynamics PhosphorImager. (*B*) Standard elongation assays (as in *A*) were carried out upon pSAB12. No activator (lanes *1,4*), the GAL4 DNA-binding domain (amino acids 1–94; lanes *2,5*), or the GAL4–HSF fusion protein (lanes *3,6*) was preincubated (at 1.2-fold molar excess over sites) with template for 15 min, and remained present throughout the transcription. In lanes *4–6*, one unit of the hSWI/SNF fraction was preincubated with assembled templates in the presence of 4 mM ATP, and remained present during the final elongation step. The hSWI/SNF fraction was absent from lanes *1–3*. These experiments are quantitated in Fig. 6.

we next addressed whether the HSF activation domain itself was necessary for this relief. To do this, we tested the GAL4 DNA-binding domain alone (amino acids 1–94 of the GAL4 protein) to see whether it could promote a similar effect in the presence or in the absence of the hSWI/SNF fractions (Fig. 5B). Under both conditions, maximal readthrough of the pause required the HSF activation domains (cf. lanes 2 and 5 with lanes 3 and 6). Data from these and other experiments were quantified by determining the ratio (full length transcript)/(+46 to +49 paused transcript + full length transcript) using a PhosphorImager, and this ratio was plotted at different concentrations of hSWI/SNF in the reaction (Fig. 6). There was no significant effect of hSWI/SNF fractions on readthrough in the absence of an activator at any concentration. Maximal effects on readthrough required both GAL4–HSF and the hSWI/SNF fraction; under these conditions, readthrough increased 10-fold over that seen without activator. GAL4(1–94) had a slight effect on readthrough that was significantly lower than the effect of GAL4–HSF. This small effect is consistent with the previously documented ability of GAL4(1–94) to stimulate transcription weakly on nucleosomal templates (Workman et al. 1991b; Croston et al. 1992). We conclude that GAL4–HSF can increase readthrough of a nucleosome-dependent pause in this in vitro system.

GAL4–HSF and hSWI/SNF fractions do not affect pausing on naked DNA templates

The effect of GAL4–HSF on pausing on the nucleosomal template might reflect a general ability of this activator to effect elongation or might be specific to regulation of elongation on nucleosomal templates. We examined elongation of transcription on the naked *hsp70* template and detected several positions where RNA polymerase pauses transiently, including a pause at +46 to +49 that had a half-life of ~3 min (Fig. 7). We found no effect of GAL4–HSF or hSWI/SNF fractions on the extent of pausing on the naked *hsp70* promoter and conclude that the effects we observe upon pausing are specific to nucleosomal templates.

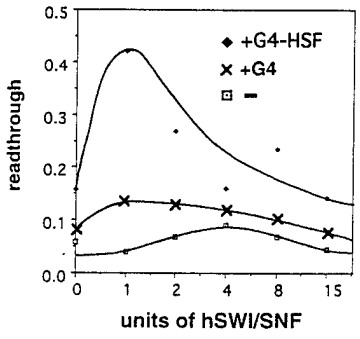

Figure 6. The effects of GAL4–HSF and the GAL4 DNA-binding domain are graphed as a function of the amount of hSWI/SNF present in the reaction. Transcriptional assays were performed exactly as in Fig. 4 but with the amount of hSWI/SNF indicated along the *x*-axis present during the final elongation step. Readthrough was quantitated as follows: (amount of +465 transcript)/(amount of +465 transcript + amount of +46 to +49 transcript). The data shown are from a single typical titration; the trends observed were verified in several other such titrations.

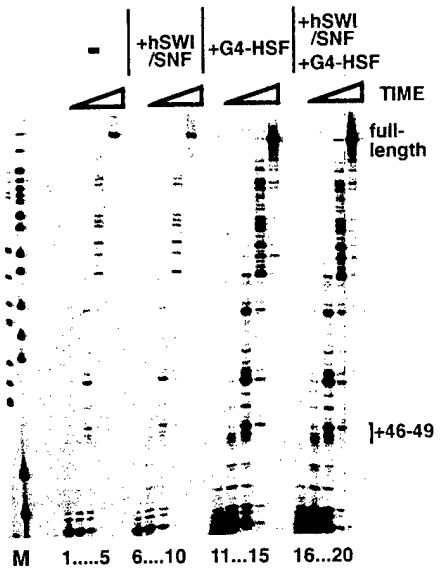

Figure 7. Effects of GAL4–HSF and hSWI/SNF upon pausing on naked DNA templates. Transcription complexes were artificially stalled at +15 on pSAB12 templates by adding only ATP, CTP, and labeled UTP. After washing, the complexes were elongated by adding all four unlabeled nucleotides and incubating for 0 min (lanes *1,6,11,16*), 1 min (lanes *2,7,12,17*), 3 min (lanes *3,8,13,18*), 10 min (lanes *4,9,14,19*), or 30 min (lanes *5,10,15,20*). In lanes *11–20,* GAL4–HSF was preincubated (at a 1.2-fold molar excess over binding sites) with templates for 15 min prior to the start of transcription reactions, and remained present throughout the reactions. In lanes *6–10* and *16–20,* 1 unit of the hSWI/SNF fraction was incubated with artificially stalled templates in the presence of 4 mM ATP for 15 min prior to the addition of all four nucleotides, and remained present throughout the elongation.

Discussion

The primary conclusions from this work are that nucleosome formation greatly enhances a transcriptional pause on the human *hsp70* gene (Figs. 2 and 4) in vitro and that the HSF1 transcriptional activation domain can relieve this pause (Fig. 5). The effect of the activation domain on pausing is seen only on a nucleosomal template; therefore, our data imply that a novel and important aspect of activation domain function is to decrease the inhibitory effect of nucleosomes on elongation of RNA polymerase. Previous studies have demonstrated that total transcription is stimulated to a significantly greater extent by activation domains on nucleosomal templates as compared to naked templates (Workman et al. 1991b; Croston et al. 1992). This difference has been attributed to an ability of activation domains to alleviate a nucleosome-dependent inhibition of pre-initiation complex formation (for review, see Workman and Buchman 1993). The ability of activation domains characterized here to alleviate nucleosome-dependent inhibition of elongation complements this work.

Nucleosomes affect pausing on the human hsp70 *gene*

It has been appreciated for some time that there are significant steric problems to be overcome during elongation of RNA polymerase through a nucleosome. RNA polymerase II has been shown to transcribe through nucleosomal DNA in vitro (Lorch et al. 1987; for review, see van Holde et al. 1992); however, studies with SP6 polymerase suggest that the nucleosome is displaced in a process that appears to involve direct transfer to different DNA sequences on the same template (Clark and Felsenfeld 1992; Studitsky et al. 1994). From these data, it seemed reasonable that nucleosomes might inhibit the rate of transcriptional elongation, and nucleosome formation has been shown to increase pausing of RNA polymerase II in vitro (Izban and Luse 1991).

We show here that nucleosome formation has a particularly dramatic effect on formation of a paused polymerase at nucleotides +27 and +46 of the human *hsp70* transcribed region. There is a pause that lasts ~3 min at the latter site on naked DNA (Fig. 7). On nucleosomal templates, these pauses last for ≥6 hr (the longest timepoint we have examined), so the duration of pausing is increased ≥100-fold by formation of the template into nucleosomes. We have used permanganate footprinting to show that one of these pauses is similar to one in vivo (Fig. 1). This region of the human *hsp70* template is nucleosomal in vivo (Fig. 3), consistent with the hypothesis that nucleosomes might contribute to formation of this pause, in vivo as well as in vitro. Such nucleosomes need not even be positioned precisely to facilitate specific pausing. Izban and Luse (1991) and O'Neill et al. (1992) have found that nucleosomes greatly enhance intrinsic DNA pausing sites. Studitsky et al. (1995) have reached similar conclusions, although they believe that this enhancement is confined to a particular region of the nucleosomal core. Our data concerning enhancement of the +46 to +49 pause is consistent with this previous work. Furthermore, pausing on the *hsp70* gene is conserved between humans and *Drosophila,* and promoter–proximal pauses are seen on several other mammalian and *Drosophila* genes (Rougvie and Lis 1988; Spencer and Groudine 1990). This fact raises the possibility that the observations we have made here concerning the role of nucleosomes on inducing pauses might be more general.

Relief of pausing by activation domains

Numerous studies in *Drosophila* and in mammalian tissue culture have led to the hypothesis that activators might regulate pausing of RNA polymerase II. For example, characterization of RNA polymerase occupancy of the *Drosophila hsp70* promoter demonstrated that a polymerase complex is paused proximal to the promoter prior to induction and that induction must cause some release of the pause (Rougvie and Lis 1988). Recently, it has been shown that activators or enhancers can decrease pausing on transfected or injected promoters (Yankulov et al. 1994; Krumm et al. 1995).

The data presented here demonstrate an effect of activators on pausing in vitro and imply that chromatin structure plays an important role in this regulation: Nucleosome formation creates an increased block to tran-

scriptional elongation, and activation domains are able to suppress this block. Activators might relieve the block to elongation through interaction with the general transcription machinery, through a direct destabilization of nucleosomal structure in transcribed regions, or via both mechanisms. It has been proposed that promoter–proximal pausing like that observed on *hsp70* is caused by contacts between the elongating RNA polymerase and general transcription factors that remain bound to the TATA box (Usheva et al. 1992; Lis and Wu 1993). It is possible that in the system described here, such contacts are necessary for pausing but that the additional constraint imposed by a nucleosome is also required. In this scenario, the effect of the activator on the contacts between the general transcription factors and RNA polymerase suffices to increase elongation; however, maximal effects on nucleosomal templates require additional activities that are present in the hSWI/SNF fractions. It is possible that these activities are simply facilitating activator occupancy, but we disfavor this possibility because vast excesses of activator cannot suppress the enhancement of elongation afforded by the hSWI/SNF fraction and because protein gels of washed templates show that saturating levels of activator are stably associated with the template (data not shown).

A role for nucleosome-disrupting activities in regulation of pausing

It was necessary to supplement our in vitro system with fractions that have hSWI/SNF activity to achieve maximal effects of the activator on elongation. These fractions have been shown previously to contain an ATP-dependent nucleosome-disrupting activity (Imbalzano et al. 1994; Kwon et al. 1994). Whereas the effect of these fractions on elongation requires hydrolyzable ATP, and two separate highly enriched fractions (hSWI/SNF A and B) both enhance elongation, human SWI/SNF fractions are not homogeneous, so we cannot rigorously conclude that the nucleosome disruption activity itself is responsible for the effects on elongation. We can rule out the possibility that previously characterized elongation factors are responsible for the relief of pausing, though, because the effects of the hSWI/SNF fractions on elongation require hydrolyzable ATP (data not shown) and there is no effect of these fractions upon elongation on naked DNA (Fig. 7). Neither of these observations is true of known elongation factors. The hSWI/SNF fractions are not sufficient to increase elongation on nucleosomal templates because they have no effect in the absence of activator (Figs. 5 and 6).

These observations are complicated further by recent evidence suggesting that there are likely to be multiple complexes with ATP-dependent nucleosome disruption activity in the cell (Kwon et al. 1994; Tsukiyama et al. 1995). Our hSWI/SNF fractions contain some but not all of these complexes, so the work presented here is most simply interpreted as suggesting that some remodeling activity is important to facilitate relief of nucleosome-dependent pausing. Moreover, the results above also argue against a simple single-cause model for the regulation of promoter–proximal pausing. It is clear that nucleosomes are absolutely required to achieve pausing, yet chromatin-reorganizing factors alone are unable to release pausing; hence, activators probably do more than just recruit such factors. Transcriptional activators will release the pause, yet it does not appear that chromatin-reorganizing factors are required to facilitate their occupancy under our assay conditions. It is most consistent with the data, therefore, to argue that activators and the activities present in the hSWI/SNF fraction are acting differently in a regulatory mechanism, but that both activities are necessary to achieve full release of pausing.

Materials and methods

Templates for in vitro transcription

All transcriptions were done using templates derived from the vectors pSAB8, pSABmix, pSAB12, or pML5–4NR. pSAB8 contains human *hsp70* sequences from −122 to +567 cloned *Hin*dIII–*Hin*dIII (−122) and *Bgl*II–*Bam*HI (+567) into the pUC18 polylinker. Three *hsp70* point mutations were engineered by PCR to eliminate guanosine residues between +1 and +14. (The modified sequence reads +1-TAACTCCTATCCTG-+14.) pSABmix is a mixture of plasmids identical to pSAB8, except that they contain modified *hsp70* sequences only to +335, followed by zero, one, two, or three 20-bp linkers of sequence 5′-GATCTGGCGTAATTCGGGTT-3′. pSAB12 is a pUC18-based vector that contains five GAL4 17-mer binding sites (Giniger et al. 1985) joined to the human *hsp70* gene from −35 to +567. As in pSAB8, three *hsp70* point mutations were engineered by PCR to eliminate guanosine residues between +1 and +14. pML5–4NR (Izban and Luse 1991) contains the adenovirus major late promoter from −171 to +37 fused to four 150-bp repeats from the bacterial chloramphenicol acetyltransferase (CAT) gene. (Again, point mutations have been engineered to eliminate all G residues between +1 and +15.) It was provided to us by Dr. Donal Luse (Cleveland Clinic Foundation Research Institute, OH).

Prior to in vitro transcription, these plasmids were linearized, tethered to avidin–acrylic beads at an *Eco*RI site 2.6 kb upstream of the *hsp70* promoter, and cut at +445 (pSAB8, pSAB12) or +335, +355, +375, and +395 (pSABmix) or +3.1 kb (pML5–4NR) to produce templates for runoff transcripts of these lengths.

In vitro transcription and nucleosome assembly

To preform RNA polymerase ternary complexes and to end-label the transcripts (Figs. 2–7), we used a protocol adapted from Izban and Luse (1991). We incubated 0.5 μg of bead-bound template for 1 hr at 30°C in a 25-μl reaction, including 2 mM $MgCl_2$, 0.6 mM ATP and CTP (Ultrapure; Pharmacia), 0.5 mM [α-^{32}P]UTP (800 Ci/mmole; New England Nuclear), 20 units of RNAsin (Promega), and 15 μl of total HeLa transcription factors and buffer D (Buffer D is 100 mM KCl, 20% glycerol, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF; factors included DE52-fractionated TFIIA and P11-fractionated other general transcription factors (Sumimoto et al. 1990). The amount of factors included in each reaction was empirically determined as that which gave maximal transcription on naked DNA. In Figures 2–4, 3 μl of a P11 0.8 M fraction and 2 μl each of a P11 0.5 M fraction and DE52-fractionated TFIIA were used. In Figures 5–7, a different preparation of the same three frac-

tions was used, in amounts of 4, 3, and 3 μl, respectively.] Templates were then pelleted and washed once with 50 μl of 0.6× buffer D plus 1 mM $MgCl_2$ and 1% Sarkosyl, and once with 50 μl of 0.6× buffer D plus 2 mM $MgCl_2$.

To preform preinitiation complexes at +1 rather than elongation complexes at +15 (Fig. 4B), we incubated 0.5 μg of bead-bound template for 1 hr at 30°C in a 25-μl reaction including 2 mM $MgCl_2$ and 15 μl of total HeLa transcription factors and buffer D. Templates were then pelleted and washed twice with 50 μl of 0.6× buffer D plus 2 mM $MgCl_2$. After nucleosome assembly (see below), the transcripts in these preinitiation complex reactions were endlabeled by incubating bead pellets for 1 hr at 30°C in a 25-μl reaction including 2 mM $MgCl_2$, 0.6 mM ATP and CTP (Ultrapure; Pharmacia), 0.5 mM [α-^{32}P]UTP (800 Ci/mmole; New England Nuclear), 20 units of RNAsin (Promega), and 15 μl of buffer D. The reactions were then washed once with 50 μl of 0.6× buffer D plus 2 mM $MgCl_2$.

To assemble templates into nucleosome cores after stalling transcription complexes on them (Figs. 2–6), the washed reactions were resuspended in 25 μl of buffer D plus 2 mM $MgCl_2$ and 20 units of RNasin. To this, we added 50 μl of *Xenopus laevis* heat-treated assembly extract and 2 μg of purified HeLa core histones (Workman et al. 1991a). The reactions were incubated for 1.5 hr at 30°C, then spun down, and washed with 50 μl of 0.6× buffer D plus 2 mM $MgCl_2$. [The heat-treated assembly extract (dHTE) used in this study was made as directed in Workman et al. (1991a) but was subsequently desalted over a P6DG column (Bio-Rad) to remove endogenous nucleotides. Extract was mixed with histones and preincubated at room temperature for 15 min prior to use in the reactions described above.]

To continue the elongation, either immediately after assembly (Figs. 2–6), after transcript endlabeling (in the case of reactions with a stalled preinitiation complex, Fig. 4B), or after restriction enzyme digestion (Fig. 3A; see method below), washed reactions were incubated for 1 hr at 30°C in a 25-μl reaction including 2 mM $MgCl_2$, 0.6 mM ATP, CTP, GTP, and UTP (Ultrapure; Pharmacia), 20 units of RNAsin (Promega), and 15 μl of buffer D (modified to contain 250 mM KCl, so that the final KCl concentration was 150 mM to inhibit reinitiation). The reactions were stopped with 50 μl of stop solution [67 mM NaOAc (pH 5), 6.7 mM EDTA, 0.33% SDS, 0.66 mg/ml of tRNA]. This basic protocol was modified slightly for reactions containing activators or hSWI/SNF fractions. The modifications are described later in this section.

Completed reactions were phenol-extracted, ethanol-precipitated, and analyzed on a 7.5% acrylamide/7 M urea/1× TBE sequencing gel. End-labeled marker DNA (labeled M in figures) was either *Msp*I-digested pBR322 DNA (New England Biolabs) or Boehringer Mannheim Marker V. Gels were exposed and quantitated on a Molecular Dynamics PhosphorImager.

Restriction test for template assembly

Transcription reactions were begun by transcription complex assembly followed by nucleosome assembly, exactly as described above. They were then resuspended in 25 μl of digestion buffer (0.6× buffer D with 7 mM $MgCl_2$), and 20 units of the appropriate restriction enzyme (from New England Biolabs) was added. Reactions were digested for 2 hr at 30°C and then washed once with 0.6× buffer D plus 1% Sarkosyl and 1 mM $MgCl_2$ (a treatment that also strips nucleosomes) and once with 0.6× buffer D plus 2 mM $MgCl_2$. Elongation was then continued, as above, and reactions were stopped and analyzed as described previously. (This digestion protocol was adapted from Morse 1989.)

Activators and hSWI/SNF in in vitro transcriptions

The GAL4 DNA-binding domain (amino acids 1–94) was purified from *E. coli* as described in Chasman et al. (1989). It was 17 μM in concentration of dimer active for DNA binding, and 80% pure and 80% active relative to total protein. The GAL4–HSF protein contains amino acids 1–147 of the GAL4 DNA-binding domain fused to amino acids 202–529 of human HSF1, which has been characterized in vivo as the regulatory and activation domains (Green et al. 1995; Zuo et al. 1995). It was expressed in *E. coli* as a 6× His-tagged fusion with the aid of the pRJR1 expression vector (Reece et al. 1993) and purified over a nickel–Sepharose column (Qiagen) according to the manufacturer's directions. The resulting preparation was 4 μM in concentration of active dimer, and was 30% pure and 90% active. Both proteins were dialyzed into buffer D.

Reactions containing GAL4 or GAL4–HSF (Figs. 5–7) were begun by prebinding the proteins to their cognate DNA sites: We incubated 0.5 μg of bead-bound template for 15 min at room temperature in an 8-μl reaction including 2 mM $MgCl_2$ and a total of 5 μl of buffer D plus a 1.2 molar excess of activator relative to DNA-binding sites, or 1.4 pmole. This reaction was then increased to 25 μl in volume by supplementing with 0.6 mM ATP and CTP (Ultrapure; Pharmacia), 0.5 mM [α-^{32}P]UTP (800 Ci/mmole; New England Nuclear), 20 units of RNAsin (Promega), and 10 μl total of HeLa transcription factors and buffer D, plus $MgCl_2$ to maintain a concentration of 2 mM, and incubated for 1 hr at 30°C. Reactions were subsequently washed, assembled, and elongated as described above, but after each washing step reactions were supplemented with fresh protein at the same molar excess.

hSWI/SNF protein used in these studies (Figs. 5–7) was purified as described in Kwon et al. (1994) as far as the EconoQ column (Pharmacia), and dialyzed into buffer D. In these studies, it was added after assembly and prior to final elongation. Specifically, after nucleosome assembly and washing, transcription reactions were resuspended in a 23-μl reaction including 2 mM $MgCl_2$, 4 mM ATP, 20 units of RNAsin (Promega), and 15 μl total of buffer D (modified to contain enough KCl that the concentration in the final reaction is 150 mM to inhibit reinitiation) plus the amount of hSWI/SNF fraction indicated in Figures 5–7. Reactions were then incubated for 20 min at 30°C, whereupon they were supplemented with 2 μl of the other three nucleotides (to 0.6 mM concentration). Subsequent transcriptional elongation and gel analysis of transcription products proceeded as described above.

Permanganate footprinting

Fifty milliliters of log-phase HeLa spinner cells were harvested, washed with PBS (pH 7.4), and resuspended in 1 ml of PBS to a final concentration of 1×10^7 cells/ml. In experiments in which heat-shocked cells were examined, log-phase HeLa cells were incubated at 43°C for 1 hr. Cells were then washed with prewarmed PBS (pH 7.4), and resuspended at the same density. One-tenth volume of fresh 0.2 M $KMnO_4$ was added, and reactions were incubated for 2 min at room temperature. Reactions were quenched with 1.5 volume of lysis solution (100 mM NaCl, 10 mM Tris at pH 7.8, 25 mM EDTA, 0.5% SDS, 1 M β-mercaptoethanol, 200 mg/ml of proteinase K) and incubated for 4 hr at 50°C. They were then deproteinized by extraction once with equilibrated phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform, and NaOAc was added to a final concentration of 0.3 M.

Alternatively, genomic DNA (obtained by lysis and deproteinization as described in the previous paragraph, but without

permanganate treatment) was resuspended at 1 mg/ml. One-tenth volume of fresh 0.2 M $KMnO_4$ was added, and the reaction was incubated for 2 min at room temperature. The reaction was quenched with 0.5 volume of DMS stop solution (1.5 M NaOAc at pH 7, 1 M β-mercaptoethanol).

In vivo and in vitro reactions were both precipitated once with ethanol, washed with 70% ethanol, and briefly dried. They were then resuspended in 0.3 ml of 10% piperidine, incubated for 30 min at 90°C, and lyophilized. Dried reactions were resuspended in 100 ml of H_2O, and lyophilized twice more, and precipitated three times with 0.3 M NaOAc and 2.5 volumes of ethanol before a final resuspension in TE at ~2 mg/ml of DNA.

Cleavages were visualized by LMPCR. LMPCR was performed according to the method of Mueller and Wold (1989), with 6 μg of genomic DNA per LMPCR reaction. Primers used to visualize the coding strand were primer 1, 24-mer (+186 to +163 relative to the start of transcription); primer 2, 26-mer (+154 to +129); primer 3, 27-mer (+147 to +121). Primers used to visualize the noncoding strand were primer 1, 23-mer (−106 to −84); primer 2, 28-mer (−73 to −46); primer 3, 28-mer (−65 to −38).

Southern hybridization

Nine plates of semiconfluent Hela cells were trypsinized from their plates, washed in PBS, and resuspended in 20 ml of buffer L [5 mM PIPES (pH 8), 85 mM KCl, 1 mM $CaCl_2$, 5% sucrose] with 0.5% NP-40, and incubated for 10 min on ice. Resultant nuclei were washed twice in detergent-free buffer L, and then resuspended in 2 ml of buffer M [15 mM Tris (pH 7.5), 15 mM NaCl, 60 mM KCl, 15 mM 2-mercaptoethanol, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 0.34 M sucrose]. Nuclei were adjusted to 1 mg/ml by monitoring 260-nm optical absorbance of a small amount diluted in 2 M NaCl. Aliquots of nuclei (0.4 ml) were briefly warmed to 30°C and treated with 30–600 units of micrococcal nuclease (Sigma) for 3 min. Reactions were then quenched with 1 ml of stop buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 15 mM EDTA, 0.3% SDS) and treated for 1 hr at 37°C with 50 μg of RNase A (Sigma). Reactions were then extracted twice with equilibrated phenol, and precipitated twice with 0.3 M NaOac and ethanol.

Thirty micrograms of micrococcal nuclease-treated DNA was loaded into each lane of a 1.5% agarose gel. Marker lanes included 2 μg of *Msp*I-digested pBR322 DNA from New England Biolabs. The gel was blotted to New England Nuclear Genescreen membrane according to the instructions of the manufacturer. Nucleic acids were cross-linked to the membrane with ultraviolet light using a Stratalinker (from Stratagene), also according to the manufacturer's directions.

The blot was probed with ~10 ng of *Bam*HI–*Sac*II *hsp70* restriction fragment cut from pSAB8, following protocol I of the membrane manufacturer's instructions. (This probe has been shown previously to recognize a single band on blots of appropriately restriction-digested HeLa DNA.) The probe was labeled to a specific activity of 4×10^9 cpm/μg with a Boehringer Mannheim Random Priming Kit. The resultant labeled blot was exposed for 5 days on Kodak XAR film at −70°C with an intensifying screen.

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RNA Polymerase II Holoenzyme Contains SWI/SNF Regulators Involved in Chromatin Remodeling

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Summary

The RNA polymerase II holoenzyme contains RNA polymerase II, a subset of general transcription factors and SRB regulatory proteins. We report here that *SWI* and *SNF* gene products, previously identified as global gene regulators whose functions include remodeling chromatin, are also integral components of the yeast RNA polymerase II holoenzyme. The SWI/SNF proteins are components of the SRB complex, also known as the mediator, which is tightly associated with the RNA polymerase II C-terminal repeat domain. The SWI/SNF components provide the holoenzyme with the capacity to disrupt nucleosomal DNA and thus facilitate stable binding of various components of the transcription initiation complex at promoters.

Introduction

Regulation of class II genes involves a complex interplay among gene-specific activators and cofactors, the general transcription apparatus, and chromatin. Gene-specific activators bind to promoters and stimulate transcription, at least in part, by binding and recruiting the general transcription apparatus (Chen et al., 1994; Hengartner et al., 1995; Ingles et al., 1991; Lin et al., 1991; Xiao et al., 1994; reviewed by Tjian and Maniatis, 1994; Sheldon and Reinberg, 1995; Emili and Ingles, 1995; Carey, 1995). Chromatin structure can affect the transcriptional activity of genes by blocking access of the transcription apparatus to promoters (Knezetic and Luse, 1986; Bresnick and Felsenfeld, 1993; Felsenfeld, 1992; Lorch et al., 1988; Workman and Roeder, 1987). The SWI and SNF proteins are global regulators that function by antagonizing repression mediated by nucleosomes, altering chromatin structure to facilitate binding of the transcription apparatus (Côté et al., 1994; Hirschhorn et al., 1992; Imbalzano et al., 1994; Kwon et al., 1994; reviewed by Carlson and Laurent, 1994; Peterson and Tamkun, 1995; Winston and Carlson, 1992). It is not yet clear how the SWI/SNF proteins are targeted to promoters, although some gene-specific activators may interact directly with these proteins (Yoshinaga et al., 1992).

Genetic and biochemical studies in yeast indicate that the form of the transcription initiation apparatus generally responsible for mRNA synthesis in vivo is an RNA polymerase II holoenzyme (Barberis et al., 1995; Hengartner et al., 1995; Kim et al., 1994; Koleske and Young, 1994; Thompson and Young, 1995; reviewed by Carey, 1995; Emili and Ingles, 1995; Koleske and Young, 1995). This megadalton-sized complex contains RNA polymerase II, general transcription factors, and additional components called suppressor of RNA polymerase B (SRB) regulatory proteins. The SRB proteins are a hallmark of the holoenzyme. The genes encoding the nine known SRB proteins were identified through a selection for factors involved in transcription initiation by RNA polymerase II in vivo, and all are required for normal yeast cell growth. Essentially all of the SRB protein in cells is tightly associated with the holoenzyme, while approximately 80% of RNA polymerase II and general transcription factors are found independent of this complex (Koleske and Young, 1995). Experiments with temperature-sensitive *SRB* mutants indicate that the RNA polymerase II holoenzyme is the form of the transcription initiation apparatus employed at the majority of class II promoters in vivo (Thompson and Young, 1995). Other experiments have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995).

The yeast *SWI* genes were first identified as positive regulators of HO transcription (Stern et al., 1984), and *SWI1*, *SWI2*, and *SWI3* were later shown to be required for the activation of a broad spectrum of inducible genes in vivo (Peterson and Herskowitz, 1992; Yoshinaga et al., 1992). Similarly, the *SNF* genes were originally identified as positive regulators of *SUC2* (Neigeborn and Carlson, 1984), and *SNF2*, *SNF5*, and *SNF6* were subsequently found to be essential for activation of a diverse set of inducible genes (Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992). Further study revealed that *SWI2* and *SNF2* are the same gene. Genetic evidence indicated that the *SWI* and *SNF* genes are involved in similar processes in gene activation (Carlson and Winston, 1992). Indeed, the discovery that SWI1, SWI2/SNF2, SWI3, SNF5, SNF6, and SNF11 proteins copurify in a large complex confirmed that the *SWI/SNF* gene products function together (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994; Treich et al., 1995). Genetic and biochemical evidence implicated the SWI/SNF proteins in chromatin remodeling via nucleosome disruption (Cairns et al., 1994; Côte et al., 1994; Hirschhorn et al., 1992; Peterson et al., 1994).

Several lines of evidence led us to investigate whether SWI and SNF proteins are components of the RNA polymerase II holoenzyme, and furthermore, whether SWI/SNF proteins are components of the SRB-containing protein complex that is tightly associated with the C-terminal repeat domain (CTD) in the holoenzyme.

First, genetic evidence suggests a functional relationship between the *SWI* and *SNF* gene products and the CTD. Strains containing mutations in *SWI* genes exhibit a large number of defects similar to those due to a truncation of the RNA polymerase II CTD (Nonet et al., 1987; Peterson and Herskowitz, 1992; Peterson et al., 1991). In addition, the CTD and the *SWI/SNF* gene products show similar genetic interactions with mutations in *SIN1* and *SIN2*, genes that encode chromatin-associated proteins (Peterson and Herskowitz, 1992; Peterson et al., 1991). Second, the *SRB* gene products have functional and physical interactions with the RNA polymerase II CTD (Koleske and Young, 1995), which has been implicated in the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990). Third, the holoenzyme appears to be responsible for initiating transcription of most, if not all, class II genes in yeast, and the *SWI* and *SNF* gene products are required for transcriptional induction of a large number of genes in vivo (Thompson and Young, 1995; Peterson et al., 1991). Finally, there are perhaps a dozen polypeptides in purified yeast RNA polymerase holoenzyme that have yet to be identified.

We report here that the yeast RNA polymerase II holoenzyme contains SWI2/SNF2, SWI3, SNF5, and SNF11. The SWI/SNF proteins are components of the SRB complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the SRB/SWI/SNF complex have ATP-dependent nucleosome disruption activities previously ascribed to the SWI/SNF complex. In addition, the holoenzyme facilitates the binding of TATA box–binding protein (TBP) to nucleosomal DNA in an ATP-enhanced manner.

Results

Anti-SRB and Anti-SWI Antibodies Coprecipitate Holoenzyme

SRB regulatory proteins are found tightly and exclusively associated with other components of the RNA polymerase II holoenzyme in cell extracts. If SWI and SNF proteins are subunits of the RNA polymerase II holoenzyme, then antibodies against SRB5 should precipitate both the holoenzyme and SWI/SNF proteins from crude extracts. The results in Figure 1 show that this is indeed the case. SWI2/SNF2, SWI3, and SNF5 proteins coprecipitate with holoenzyme obtained through SRB5 immunoprecipitation. The fraction of SWI and SNF proteins immunoprecipitated from the crude extract appears to be the same as that of the SRB proteins. Control proteins introduced into the crude lysate did not coprecipitate, indicating that the immunoprecipitate was specific for the holoenzyme. When the immunoprecipitation experiment was carried out with antibody against SWI3, essentially identical results were obtained (Figure 1). The SWI/SNF and SRB proteins were immunoprecipitated from the crude extract with similar efficiency whether the immunoprecipitating antibody used was directed against SRB5 or SWI3. A control experiment with antibody against TGFβ failed to precipitate SWI/SNF or SRB proteins. These results indicate that

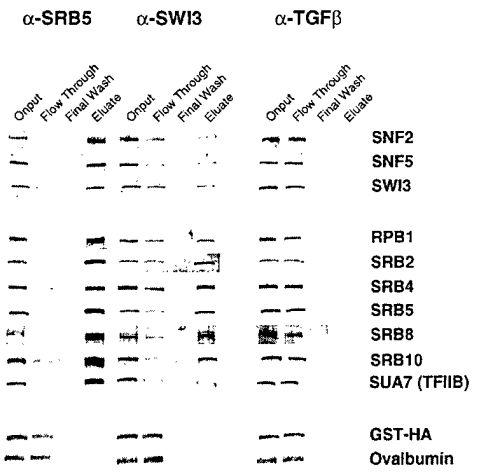

Figure 1. Immunoprecipitation of RNA Polymerase II Holoenzyme from Crude Extracts Using Anti-SRB5 and Anti-SWI3 Antibodies

Immunoprecipitations were from a crude DEAE fraction prepared as described by Hengartner et al. (1995). Immunoprecipitations were carried out with affinity-purified antibodies against SWI3, SRB5, or TGFβ. Ovalbumin and HA-tagged GST were added to each reaction prior to precipitation to serve as controls for specific immunoprecipitation; 1/50 of the onput and flowthrough, and 1/5 of the final wash and eluate were subjected to SDS–PAGE and analyzed by Western blotting using specific antibodies.

SRB and SWI/SNF proteins are tightly associated with one another.

Purified Holoenzyme Contains SWI/SNF Proteins

The immunoprecipitation results led us to investigate whether SWI and SNF proteins are components of purified yeast RNA polymerase II holoenzyme. Antibodies against selected SWI and SNF proteins were used to determine whether these proteins coelute with the RNA polymerase II holoenzyme in the final purification step of the holoenzyme. The data in Figure 2A demonstrate that SNF2/SWI2, SNF5, SWI3, and SNF11 proteins coelute with other known components of the holoenzyme and with transcription activity.

The holoenzyme contains stoichiometric amounts of RNA polymerase II, SRB proteins, and general transcription factors. To ascertain whether the SWI/SNF proteins are stoichiometric components of the holoenzyme, the amounts of SNF2 and SNF5 were estimated by Western blot analysis with various amounts of recombinant proteins as standards (Figure 2B). These data indicate that the purified RNA polymerase II holoenzyme contains approximately equimolar amounts of SNF2, SNF5, and SRB5, the latter being a standard against which other holoenzyme components have previously been compared (Koleske and Young, 1994). Since yeast cells contain between 2000 and 4000 molecules of RNA polymerase II holoenzyme, it appears that there are at least this number of SWI2/SNF2 and SNF5 molecules per cell.

SWI/SNF Proteins Are Components of CTD-Binding SRB Complexes

Genetic evidence indicates that the SRB regulatory proteins and the RNA polymerase II CTD have related functions in transcription initiation and that these involve

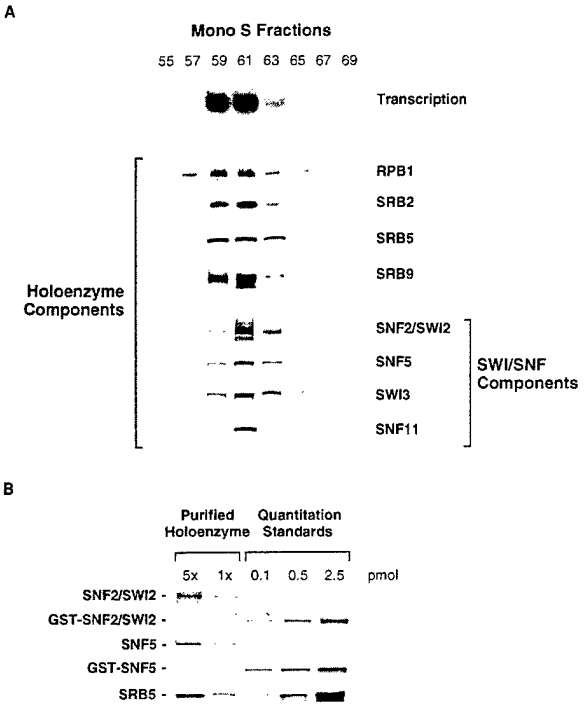

Figure 2. SWI/SNF Proteins Are Components of Purified RNA Polymerase II Holoenzyme

(A) RNA polymerase II holoenzyme eluted from a Mono S column, the last chromatographic step in the purification procedure (Koleske and Young, 1994), was analyzed for transcriptional activity and for the presence of SRB and SWI/SNF proteins by Western blotting.
(B) Quantitative Western blots were used to determine the relative amounts of SRB5 and SWI/SNF proteins in the holoenzyme. Known amounts of recombinant GST–SNF2/SWI2$_{1256-1703}$, GST–SNF5$_{1-193}$, and SRB5 were subject to SDS–PAGE and Western blot analysis along with 2.5 μl and 0.5 μl of purified holoenzyme. There are similar levels of SNF2/SWI2, SNF5, and SRB5 in the purified holoenzyme Previous studies have shown that RPB1 and other SRB proteins are equimolar in purified holoenzyme (Koleske and Young, 1994).

the response to transcriptional regulators (Allison and Ingles, 1989; Gerber et al., 1995; Scafe et al., 1990; Koleske and Young, 1995). Since the SWI and SNF proteins are also involved in activation of a wide variety of genes and since mutations in SWI and SNF genes can produce phenotypes similar to those observed with mutations in SRB genes, we investigated whether SWI and SNF proteins are associated with the SRB complex. The SRB protein complex can be released from the holoenzyme when the latter is treated with monoclonal antibodies against the CTD, and this preparation has been called mediator (Kim et al., 1994). We previously prepared a mediator complex according to the procedure of Kim et al. (1994), confirmed that it has the coactivator activity described by these investigators, and showed that the mediator contains all of the SRB proteins (Hengartner et al., 1995). When this mediator preparation was assayed for the presence of SNF2/SWI2, SNF5, and SWI3 proteins by Western blot, all three SWI/SNF proteins were found (Figure 3).

The SRB complex can also be isolated from crude extracts using a recombinant CTD column (Thompson et al., 1993). An SRB complex was purified extensively by using a recombinant glutathione S-transferase (GST)–CTD column, followed by chromatography with Mono S and Mono Q columns (Figure 4A). The SRB, SWI, and SNF proteins bind to a GST–CTD column, but not to a control GST column, indicating that they bind specifically to the CTD (Figure 4B). Sliver staining and Western blotting confirm that both a multiprotein complex containing SRB proteins and each of the three assayed SWI/SNF proteins coelute from the Mono Q col-

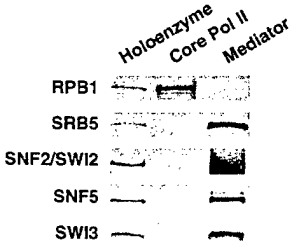

Figure 3. SWI/SNF Proteins Are Present in Mediator Purified Using 8WG16 Monoclonal Antibodies

Mediator was Western blotted along with holoenzyme and core polymerase and probed for the presence of SWI/SNF proteins. The mediator preparation was previously assayed (Hengartner et. al., 1995) and shown to have all transcriptional activities previously described (Kim et. al., 1994).

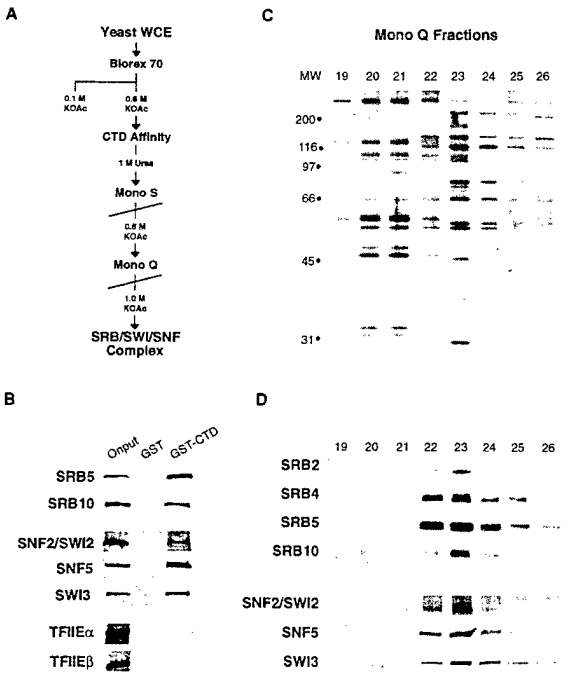

Figure 4. An SRB/SWI/SNF Complex Purified Using CTD Affinity Chromatography

(A) Schematic diagram of the purification.
(B) SRB, SWI, and SNF proteins bind specifically to a GST–CTD column. Western blot analysis of proteins eluted from a GST column and from a GST–CTD column. TFIIE was a negative control for specific retention, as it does not bind GST or GST–CTD.
(C) Silver stain of fractions across the final Mono Q column.
(D) Western blot analysis of SRB and SWI/SNF proteins across the final Mono Q column.

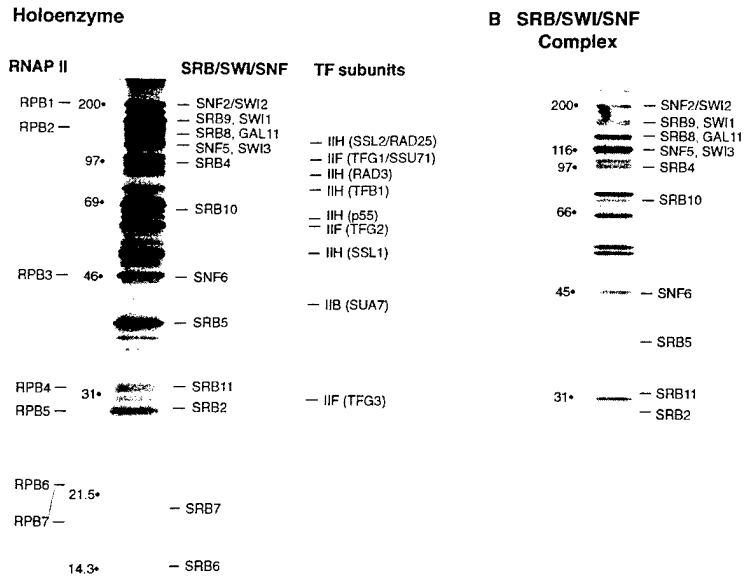

Figure 5. Components of the RNA Polymerase Holoenzyme and the SRB/SWI/SNF Complex

(A) Silver stain of purified RNA polymerase II. Bands that correspond in size to RNA polymerase core subunits, SRB, SWI, and SNF proteins, and general transcription factor IIB, IIF, and IIH subunits are indicated.

(B) Silver stain of the SRB/SWI/SNF complex. Bands that correspond in size to SRB, SWI, and SNF proteins are indicated.

umn (Figures 4C and 4D). There are approximately 25 polypeptides in this complex, and several correspond in size to previously identified SRB, SWI, and SNF proteins (Figure 5). No signals were obtained when Western blots containing the SRB/SWI/SNF complex were probed with antibodies against RNA polymerase II, TBP, TFIIB, or the TFB1 subunit of TFIIH (data not shown). These results indicate that the SRB complex is in fact an SRB/SWI/SNF complex and, furthermore, that the SWI and SNF proteins interact with the holoenzyme, at least in part through their association with RNA polymerase II CTD.

Nucleosome Disruption Activity in Holoenzyme and SRB/SWI/SNF Complex

Previous evidence that SWI1, SWI2, SWI3, SNF5, SNF6, and SNF11 gene products can be isolated as a large multisubunit complex capable of altering nucleosome structure led us to investigate whether the purified RNA polymerase II holoenzyme and the SRB/SWI/SNF complex were able to alter nucleosome structure. Mononucleosome particles were reconstituted from purified histone octamers and a DNA fragment containing two copies of an artificial phasing sequence (Shrader and Crothers, 1989). Digestion of the mononucleosomes with DNase I showed a 10 bp cleavage ladder typical of a rotationally phased nucleosome (Figure 6). Fractions in the last chromatographic step in the purification of the holoenzyme were mixed with mononucleosomes and assayed for the ability to alter nucleosome structure, which can be visualized by changes in the accessibility of the nucleosome to DNase I cleavage. Figure 6A demonstrates that a nucleosome disruption activity coeluted with the RNA polymerase holoenzyme. The ability of the SRB/SWI/SNF complex to alter nucleosome structure was assayed in a similar experiment using fractions from the last step in the SRB/SWI/SNF purification (Figure 6C). The results show that nucleosome disruption activity coeluted with the SRB/SWI/SNF complex. Further analysis of the RNA polymerase II holoenzyme and SRB/SWI/SNF complex showed that the nucleosome disruption activity was ATP dependent (Figures 6B and 6D), as was previously shown for purified SWI/SNF complexes (Côté et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994). In addition, purified core RNA polymerase II showed no nucleosome alteration capability (data not shown). These data indicate that the SRB/SWI/SNF complex contributes chromatin remodeling activity to the RNA polymerase II holoenzyme.

Purified Holoenzyme and the SRB/SWI/SNF Complex Disrupts Plasmid Chromatin

To characterize further the nucleosome disruption capabilities of the holoenzyme and the SRB/SWI/SNF complex, we employed a supercoiling reduction assay (Figure 7). In this assay, chromatin is assembled onto a relaxed closed-circular plasmid that is subsequently purified by glycerol gradient centrifugation. Each assembled nucleosome introduces approximately one negative supercoil to the plasmid, which can be resolved by agarose gel electrophoresis after the removal of histones. When no protein is added to the nucleosome-assembled plasmid, it is highly supercoiled. Fractions from the last column of the holoenzyme purification (see Figure 2A) were tested for their ability to disrupt nucleosome structure and thereby reduce supercoiling in the presence of added topoisomerase I. As can be seen in Figure 7A, this activity coelutes with holoenzyme transcription activity, with the SRB and SWI/SNF proteins (see Figure 2A), and with nucleosome-core disruption activity (see Figure 6A). The supercoiling reduction activity was dependent on ATP (Figure 7A, compare fraction 61 plus and minus ATP), as has been shown for the human SWI/SNF complex (Kwon et al., 1994). Repeating the experiment using fractions from the last column of the SRB/SWI/SNF complex shows that this complex also has an ATP-dependent supercoiling reduction activity (Figure 7B).

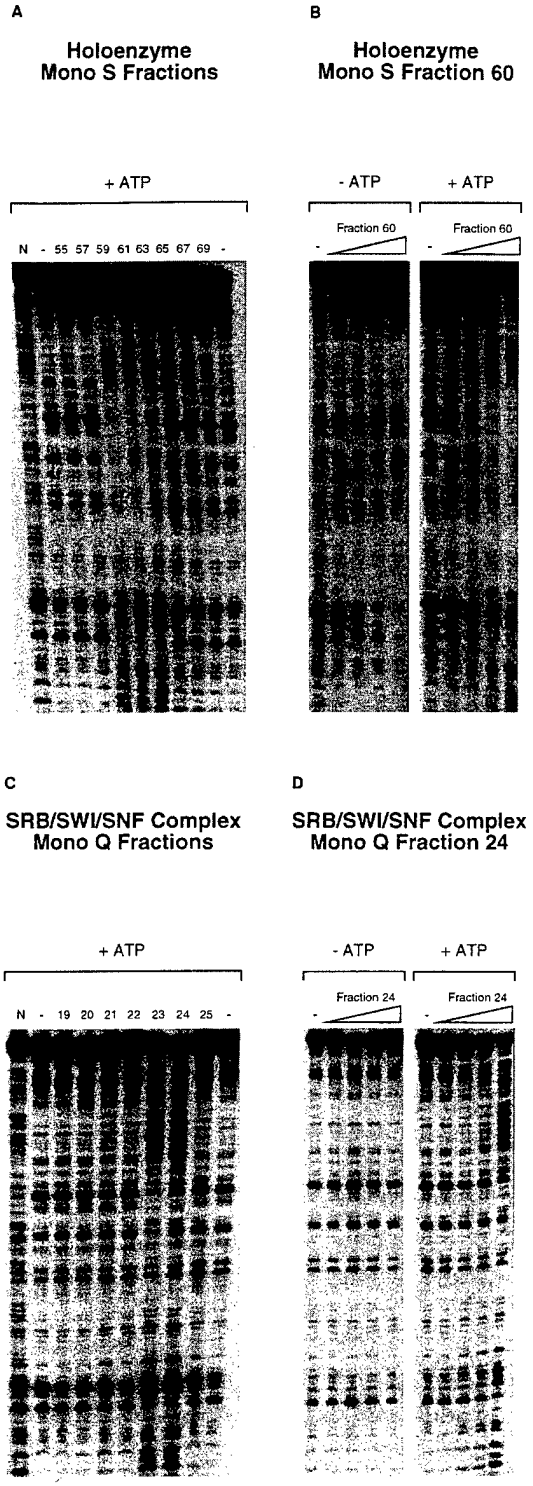

Figure 6. An ATP-Dependent Nucleosomal Disruption Activity Coelutes with the Holoenzyme and the SRB/SWI/SNF Complex

(A) Fractions from the last column of holoenzyme purification (Figure 2A) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 59–63, coincident with the peak of transcriptional activity.

(B) Purified RNA polymerase II holoenzyme (fraction 60) was titrated for activity with and without 4 mM ATP, as indicated.

(C) Fractions from the final column of the SRB/SWI/SNF complex purification (Figure 4) were assayed for nucleosomal disruption. The peak of nucleosomal disruption activity is in fractions 23 and 24, which is also where the bulk of SRB and SWI/SNF proteins elute.

(D) The SRB/SWI/SNF complex (fraction 24) was titrated for activity with and without 4 mM ATP, as indicated.

Holoenzyme Facilitates the Binding of TBP to Nucleosomes

Previous work has shown that both yeast and human SWI/SNF complexes can facilitate transcription factor binding to nucleosomal DNA containing the relevant factor-binding site (Côté et al, 1994; Imbalzano et al, 1994; Kwon et al, 1994). We tested whether the holoenzyme could increase the binding of TBP to a mononucleosome containing a TBP-binding site. With holoenzyme and ATP present, TBP and TFIIA bound to the mononucleosome at TBP concentrations of 4×10^{-6} M (Figure 8A, lane 7), while no TBP/TFIIA binding was observed in the absence of holoenzyme (Figure 8A, lane 6).

This holoenzyme-facilitated TBP binding might be caused by the stabilizing effects of the additional protein–protein and protein–DNA interactions that occur in the presence of RNA polymerase and general transcription factors, by the ATP dependent nucleosome disruption effects of SWI/SNF, or by a combination of both effects. To address this issue, we tested whether facilitated TBP binding was ATP dependent and observed partial protection of the TATA region on the mononucleosome when ATP is withheld or when ATPγS is used instead of ATP (Figure 8B, lanes 4 and 6). However, addition of ATP enhanced the TBP binding as indicated by the increased protection from DNase I cleavage over the TATA box, the extension of the footprint in the 5′ direction, and the appearance of a hypersensitive band in the 3′ direction (Figure 8B, lane 5). Thus, it appears that the holoenzyme can partially stabilize binding of TBP and TFIIA to a mononucleosome in the absence of ATP. However, the full effect of holoenzyme-facilitated TBP binding requires ATP, presumably because it involves the ATP-dependent nucleosome disruption activity of the SWI/SNF proteins.

Discussion

The RNA polymerase II holoenzyme contains *SWI* and *SNF* gene products, previously identified as global gene coactivators. The SWI and SNF proteins are components of an SRB/SWI/SNF complex, also known as the mediator, which is tightly associated with the RNA polymerase II CTD. Both the holoenzyme and the SRB/SWI/SNF complex have nucleosome disruption activities previously ascribed to the SWI/SNF complex. In addition, the holoenzyme facilitates the binding of TBP to nucleosomal DNA in an ATP-enhanced manner.

Diverse Transcriptional Activators Require SWI/SNF Function In Vivo

Mutations in *SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* cause a substantial reduction in the ability to activate transcription of a wide variety of well-studied genes in yeast cells, including *HO* (Stern et al., 1984), *SUC2* (Neigeborn and Carlson, 1984), *Ty* (Happel et al., 1991),

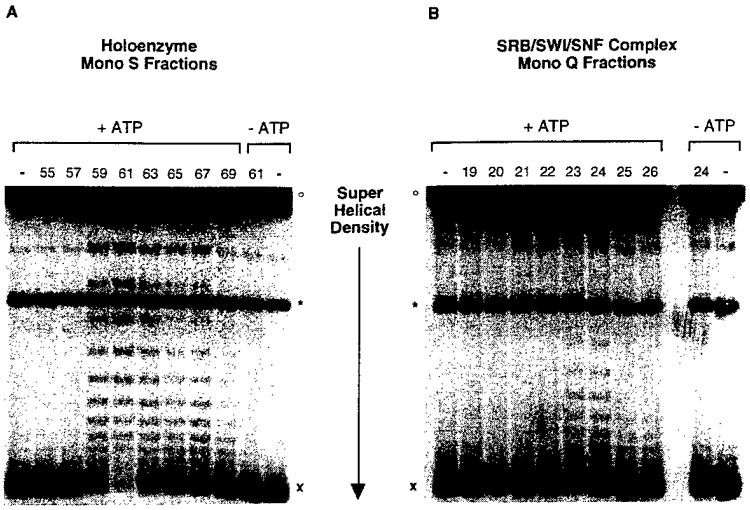

Figure 7. The Holoenzyme and the SRB/SWI/SNF Complex Reduce the Superhelical Density of Chromatin-Assembled Plasmids in an ATP-Dependent Manner

Fractions from the last column of holoenzyme purification (A) and from the last column of SRB/SWI/SNF complex purification (B) were assayed in the presence of 4 mM ATP. Peak fractions of purified holoenzyme and SRB/SWI/SNF complex were assayed with and without 4 mM ATP present as described in Experimental Procedures. The symbols o, *, and x indicate nicked circular plasmid DNA, linear DNA, and highly supercoiled circular DNA, respectively.

INO1 (Peterson et al., 1991), and *ADH1* and *ADH2* (Peterson and Herskowitz, 1992; Taguchi and Young, 1987). For example, *ADH1* and *SUC2* gene expression is reduced by about an order of magnitude in strains in which *SWI1*, *SWI2*, or *SWI3* has been deleted (Peterson and Herskowitz, 1992). Experiments with reporter constructs have revealed that the *SWI* and *SNF* gene products are required for normal responses to a variety of gene-specific activators in yeast such as GAL4, Drosophila *fushi tarazu*, mammalian glucocorticoid and estrogen receptors, and LexA–GAL4 and LexA–Bicoid fusion proteins (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992; Yoshinaga et al., 1992).

We have proposed that the RNA polymerase II holoenzyme is recruited to promoters by activators in vivo (Koleske and Young, 1994). Ptashne and colleagues have shown that recruiting a component of the SRB complex to promoters, presumably in association with the holoenzyme, suffices to obtain activated levels of transcription in vivo (Barberis et al., 1995; M. Ptashne, personal communication). Thus, evidence that LexA fusions with SWI2/SNF2, SNF5, SNF6, and SNF11 proteins are sufficient to activate transcription of a target gene in vivo (Laurent et al., 1990, 1991; Treich et al., 1995) might now be interpreted in terms of holoenzyme recruitment to the target promoter.

We propose that recruitment of the holoenzyme to a specific promoter in vivo provides a means to facilitate TBP binding, regardless of the nucleosome structure at that promoter. The holoenzyme can enhance binding of TBP and TFIIA to a mononucleosome in vitro in the absence of ATP (Figure 8), a result compatible with evidence that the polymerase and general transcription factor components of the holoenzyme provide additional protein–protein and protein–DNA interactions that should stabilize TBP binding (Buratowski, 1994). Holoenzyme-facilitated TBP binding to a mononucleosome is greater in the presence of ATP, which presumably reflects the ATP-dependent nucleosome disruption activity of the SWI/SNF proteins. These observations are consistent with the idea that SWI/SNF protein function is necessary at the subset of promoters whose chromatin structure is particularly restrictive for TBP binding.

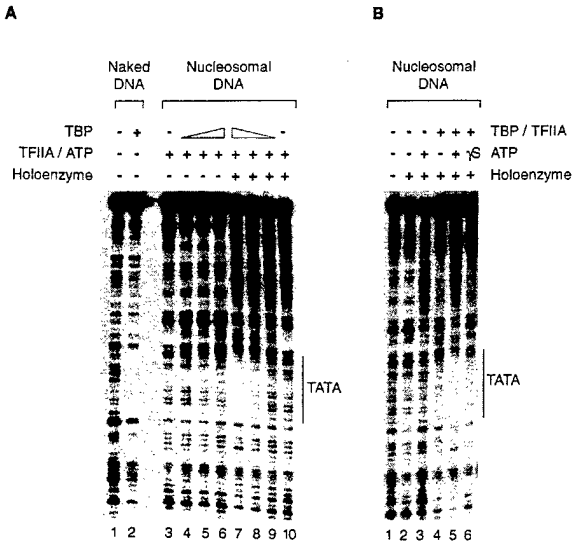

Figure 8. Holoenzyme Facilitates Binding of yTBP and yTFIIA to a Nucleosome Containing a TATA Box in an ATP-Enhanced Manner

(A) Increasing amounts of yTBP in the presence of yTFIIA and 4 mM ATP were tested for the ability to bind to a TATA box containing nucleosome with and without holoenzyme present.

(B) Nucleosomes were incubated with and without holoenzyme, 4 mM ATP or 4 mM ATPγS, yTBP and yTFIIA, as indicated.

SWI/SNF in the Holoenzyme Accounts for Previous Genetic Observations

The presence of SWI/SNF proteins in the RNA polymerase II holoenzyme and the observation that these proteins are components of a subcomplex that interacts with the RNA polymerase II CTD explain several previous observations. SWI/SNF proteins are necessary for transcription activation of many genes in yeast cells (reviewed by Winston and Carlson, 1992; Carlson and Laurent, 1994; Peterson and Tamkun, 1995); CTD truncation adversely affects the response to activators in yeast and mammalian cells (Allison and Ingles, 1989; Scafe et al., 1990; Gerber et al., 1995). Cells with RNA polymerase II CTD truncation mutations, cells with certain *SRB* mutations, and cells with *SWI1*, *SWI2*, or *SWI3*

mutations exhibit remarkably similar phenotypes (Peterson and Herskowitz, 1992; Thompson et al., 1993; Hengartner et al., 1995). The association of the SRB/SWI/SNF complex with the CTD accounts for the observation that cellular defects due to CTD mutations and *SWI* mutations can be alleviated by mutations in *SIN1* and *SIN2*, which encode an HMG1-related protein and histone H3, respectively (Kruger and Herskowitz, 1991; Peterson et al., 1991; Peterson and Herskowitz, 1992).

SRB/SWI/SNF Complex Is Associated with the RNA Polymerase II CTD

The SRB/SWI/SNF complex is tightly associated with the RNA polymerase II CTD. Independent attempts to purify various SRB proteins by column chromatography have always led us to purify the same multiprotein complex: the RNA polymerase II holoenzyme (Koleske and Young, 1994; Koleske et al., 1996; Hengartner et al., 1995; Liao et al., 1995; reviewed by Koleske and Young, 1995). Only very small amounts of SRB protein can be detected that are not associated with the holoenzyme. Two different methods have been described that permit partial purification of an SRB subcomplex. An SRB complex can be isolated using a CTD affinity column (Thompson et al., 1993) or by releasing it from a holoenzyme preparation by using monoclonal anti-CTD antibodies (Kim et al., 1994). Because neither of these preparations is homogeneous, we further purified the SRB complex obtained by CTD affinity chromatography (Figure 4). The SRB and SWI/SNF proteins coelute in the final step of the purification.

We also found that the SRB complex isolated by anti-CTD antibody release contains SWI and SNF proteins. Kim et al. (1994) demonstrated that reconstitution of the response of the holoenzyme to activators required the presence of a subcomplex that could be isolated from holoenzyme with anti-CTD antibodies, which contained SRB2, SRB4, SRB5, and SRB6, and was called the mediator of activation. Our own studies with the mediator, which was purified precisely as described by Kim et al. (1994) and has chromatographic and transcriptional properties identical to those originally described for this subcomplex, revealed that it contained all nine of the known SRB proteins (Hengartner et al., 1995). Thus, the mediator preparation and the SRB complex obtained by CTD affinity chromatography contain very similar, if not identical, complexes.

We have shown that the RNA polymerase II holoenzyme, and its SRB/SWI/SNF subcomplex, contain SWI2/SNF2, SWI3, SNF5, and SNF11. Although we do not have direct biochemical evidence that SWI1 and SNF6 are present in the holoenzyme, other genetic and biochemical data indicate that it is highly likely that SWI1 and SNF6 are also subunits of these complexes (Cairns et al., 1994; Côté et al., 1994; Laurent and Carlson, 1992; Laurent et al., 1991; Peterson and Herskowitz, 1992).

Are There Multiple SWI/SNF Complexes?

Large multisubunit complexes containing yeast SWI and SNF proteins have been purified to varying extents (Cairns et al., 1994; Côté et al., 1994; Peterson et al., 1994). Characterization of two of these preparations by Western blot analysis did not reveal the presence of SRB proteins (Peterson et al., 1994; Cairns et al., 1994). This suggests that the purification procedures employed in these studies separated the SRB and SWI/SNF proteins or that SWI/SNF complexes can exist independent of the holoenzyme.

Since SWI2/SNF2 and SNF5 are stoichiometric components of the holoenzyme and since yeast cells contain 2000–4000 molecules of RNA polymerase II holoenzyme, there are at least 2000 molecules of SWI2/SNF2 and SNF5 molecules per cell. Based on their SWI/SNF complex purification, Côté et al. (1994) estimated that there are between 50 and 150 copies of the SWI/SNF complex in yeast cells. One interpretation of these results is that most SWI/SNF protein resides in the RNA polymerase II holoenzyme, and the form of SWI/SNF complex purified by Côté et al. (1994) is the small amount of SWI/SNF protein that is in the process of assembly into holoenzyme or, alternatively, it represents a subcomplex that can be dissociated from the holoenzyme.

The ability to immunoprecipitate very similar holoenzyme complexes from crude yeast fractions using anti-SRB and anti-SWI antibodies suggests that most of the SWI/SNF protein in these fractions is associated with the holoenzyme. If the SRB and SWI/SNF proteins were in separate complexes, then the relative ratios of SRB and SWI/SNF proteins would differ in the anti-SRB and anti-SWI immunoprecipitates. However, the similar relative ratios of SRB and SWI/SNF proteins found in immunoprecipitates obtained with anti-SRB and anti-SWI antibodies (Figure 1) indicate that the SRB and SWI/SNF proteins are components of the same complex in the crude extract.

SWI/SNF Function Is Highly Conserved in Eukaryotes

SWI/SNF proteins and their functions appear to be highly conserved in eukaryotes. Putative homologs of *SNF2/SWI2* include Drosophila *brahma* and human *hbrm* and *hBRG1*, which have been cloned and implicated in transcriptional regulation (Tamkun et al., 1992; Khavari et al., 1993; Muchardt and Yaniv, 1993). A mammalian homolog of *SNF5*, called *INI1*, has also been cloned (Kalpana et al., 1994). A human SWI/SNF complex has been partially purified that has nucleosome disruption activities similar to those of the yeast SWI/SNF complex (Imbalzano et al., 1994; Kwon et al., 1994). The human SWI/SNF complex contains both hBRG1 and INI1 proteins (Kalpana et al., 1994; G. R. S., unpublished data), as would be expected based on the yeast results. Like the yeast SWI/SNF complex, the human SWI/SNF complex facilitates the binding of activators to nucleosomal DNA.

Implications for Mechanisms Involved in Transcriptional Activation

Our evidence indicates that the RNA polymerase II holoenzyme consists of core RNA polymerase II, all the general transcription factors other than TBP and TFIIA, and a CTD-associated SRB/SWI/SNF subcomplex. The presence of the SRB/SWI/SNF subcomplex in the RNA polymerase II holoenzyme has implications for the

mechanisms involved in transcription activation in vivo. Dynamic competition between chromatin proteins and an activator for a specific DNA site could be resolved in favor of the activator once the SWI/SNF-containing holoenzyme was recruited to the promoter. In this model, the activator and the holoenzyme both contribute to stable transcription initiation complex formation; the activator recruits the holoenzyme by binding to a subset of its components, and the SWI/SNF components of the holoenzyme enhance the stability of the activator–DNA interaction by destabilizing nucleosomes. This model is attractive because it provides a simple solution to the question of how SWI/SNF proteins are brought to promoters and it accounts for the coactivating and nucleosome disruption activities observed in vivo and in vitro for the SWI and SNF proteins.

Experimental Procedures

Immunoprecipitations

All immunoprecipitations were done as described (Hengartner et al., 1995). In brief, 50 μl of the DEAE 400 fraction was diluted 1:4 with modified transcription buffer (MTB) (50 mM HEPES–KOH [pH 7.3], 100 mM potassium acetate, 25 mM MgAc, 5 mM EGTA, 1 μM DTT, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 2 mM benzamidine, 2 μM pepstatin A, 0.6 μM leupeptin, and 2 μg/ml chymostatin) minus the postassium acetate. We added 4 μg of ovalbumin, 4 μg HA-GST, and 2 μg of BSA to each reaction prior to the addition of antibody and then added 0.4 μg of affinity-purified α-SRB5, 0.15 μg of affinity-purified α-SWI3, or 1.5 μg of affinity-purified α-TGFβ to the respective reactions and allowed them to incubate 2 hr at 4°C; 15 μl of goat anti-rabbit covalently linked to magnetic beads (Dynal) were then added and incubated for 1 hr at 4°C with constant agitation. Beads were precipitated with a magnet and washed three times in 200 μl of MTB buffer. The final wash contained no NP-40. Proteins were eluted off the magnetic beads by boiling in 20 μl of sample buffer.

Western Blotting

All Western blots were performed as described (Koleske and Young, 1994). Proteins were detected with the following antibodies: SRB2, SRB4, SRB5, SRB6 (Thompson et al., 1993), SRB8, SRB9 (Hengartner et al., 1995), SRB10, SRB11 (Liao et al., 1995), SWI2/SNF2, SNF5 (gift of B. Laurent), SWI3 (gift of C. Peterson), SNF11 (gift of I. Treich and M. Carlson), TFIIEα, and TFIIEβ (C. J. W. and R. A. Y., unpublished data). Quantitative Western blots were performed as described (Koleske and Young, 1994). Recombinant standards were SRB5 (Thompson and Young, 1995), GST–SNF2/SWI2$_{1256-1703}$, and GST–SNF5$_{1-193}$ (gifts of B. Laurent). GST proteins were purified as described (Smith and Johnson, 1988). Concentrations of recombinant proteins were determined using a colorimetric assay (Bio-Rad) with bovine serum albumin as a standard.

Purification of Holoenzyme and Mediator

Holoenzyme was purified as described (Koleske and Young, 1994). Transcription assays for holoenzyme were done as described (Koleske and Young, 1994). Mediator was purified as described (Hengartner et al., 1995).

SRB/SWI/SNF Complex Purification

Whole-cell extract was prepared from Red Star yeast as described (Thompson et al., 1993). We centrifuged 1.2 liters of the ammonium sulfate pellet for 30 min at 5,000 rpm in an RC3B centrifuge (Sorvall). The pellet was resuspended in 900 ml of buffer A (20 mM K-HEPES [pH 7.6], 1 mM EDTA, 1 mM DTT, 20% glycerol, and protease inhibitors [Thompson et al., 1993]). The suspension was centrifuged again for 30 min at 5,000 rpm in an RC3B centrifuge (Sorvall). The supernatant was mixed with 200 g (dry) of BioRex 70 and stirred for 20 min. The suspension was packed into a column with a 5 cm diameter and washed with 1.5 liters of buffer A plus 100 mM KOAc. Bound proteins were eluted with buffer A plus 600 mM KOAc. Fractions containing protein were pooled, frozen in liquid nitrogen, and stored at −70°C until use. Eluates from two BioRex columns (320 ml, 1.0 g of protein) were thawed and pooled; 320 ml of buffer A plus 2% Triton X-100 were added, and the mixture was centrifuged for 30 min at 12,000 rpm in a GSA rotor (Sorvall). The supernatant was loaded onto a 15 ml CTD affinity column prepared as described (Thompson et al., 1993) at a flow rate of 200 ml/hr. The column was washed with 100 ml of buffer A plus 300 mM KOAc plus 1% Triton X-100, or 100 ml of buffer A plus 300 mM KOAc. Bound proteins were eluted with buffer A plus 300 mM KOAc plus 1 M urea at a flow rate of 25 ml/hr. Fractions containing protein (3.7 mg) were pooled, frozen in liquid nitrogen, and stored at −70°C. The CTD column was equilibrated with buffer A plus 300 mM KOAc plus 1% Triton X-100, and the flowthrough was loaded again. The column was washed and eluted as before. Fractions containing protein (1.8 mg) were pooled, frozen in liquid nitrogen, and stored at −70°C. The CTD eluates were pooled, diluted with 1.5 vol of buffer A plus 0.01% NP-40, and centrifuged for 10 min at 17,000 rpm in an SS-34 rotor (Sorvall). The supernatant was loaded onto a Mono S HR 5/5 (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 3 ml of buffer A plus 120 mM KOAc plus 0.01% NP-40. Bound proteins were eluted with a 20 ml gradient of buffer A plus 0.01% NP-40 from 120 mM to 1000 mM KOAc. Fractions were frozen in liquid nitrogen and stored at −70°C until use. Fractions containing SRB4 and SRB5 as assayed by Western blotting were pooled and diluted with 2 vol of buffer B (20 mM Tris OAc [pH 7.6] plus 20% glycerol plus 1 mM DTT plus 0.01% NP-40 plus protease inhibitors). The mixture was centrifuged for 5 min in a microcentrifuge. The supernatant was loaded onto a Mono Q HRR 5/5 column (Pharmacia) at a flow rate of 0.3 ml/min. The column was washed with 1 ml of buffer B plus 200 mM KOAc. Bound proteins were eluted with a 40 ml gradient of buffer B from 200 mM to 2000 mM KOAc. The yield of SRB complex was approximately 100 μg. We analyzed 1 μl of each fraction by silver staining and 7.5–10 μl of each fraction by Western blotting.

Nucleosomal Disruption and Facilitated Transcription Factor Binding Assays

The PH MLT (Figure 6) or PH MLT(+3) (Figure 8) restriction fragments were assembled into rotationally phased mononucleosome particles, purified by glycerol gradient centrifugation, and assayed as described (Imbalzano et al., 1994). At the nucleosome concentrations and reaction conditions employed in this and previous studies, nucleosomes were determined to be stable on the basis of resistance to micrococcal nuclease, the appearance of a 10 bp repeat pattern upon DNase I digestion, and exhibition of reduced mobility upon electrophoresis in native polyacrylamide gels. We have not observed the appearance of free DNA due to nucleosome dissociation in any of our experiments.

In Figure 6, holoenzyme fractions were the same as those used in Figure 1A. In Figure 6A, 0.3 μl of each fraction was assayed in the presence of 4 mM ATP. For the titration of holoenzyme, 0 μl, 0.015 μl, 0.05 μl, 0.15 μl, and 0.5 μl of fraction 60 was used, respectively, with and without 4 mM ATP as indicated. SRB/SWI/SNF fractions were the same as those used in Figures 3C and 3D. In Figure 6C, 1.7 μl of each fraction was assayed in the presence of 4 mM ATP. For the titration in Figure 6D, 0 μl, 0.07 μl, 0.2 μl, 0.7 μl, and 2.0 μl of fraction 24 was used, respectively, with and without 4 mM ATP as indicated.

For Figure 8, binding of yeast TBP (yTBP) and yTFIIA to nucleosomes containing the PH MLT(+3) restriction fragment was performed as previously described (Imbalzano et al., 1994). In Figure 8A, all reactions contained 4 mM ATP. Following a 30 min incubation at 30°C in the presence or absence of holoenzyme (as indicated), increasing amounts of yTBP in the presence of yTFIIA were added. TBP concentrations were 0 (lanes 1, 3, and 10), 0.04 μM (lanes 2, 4, and 9), 0.4 μM (lanes 5 and 8), and 4 μM (lanes 6, 7). yTFIIA (1.5 μM) was also added to all reactions. In Figure 8B, reactions were treated with holoenzyme, alone (lanes 2 and 4), in the presence of 4 mM ATP (lanes 3 and 5), or in the presence of 4 mM ATPγS (lane 6) for 30 min at 30°C, followed by addition of 4 μM yTBP in the presence of 1.5 μM yTFIIA.

Recombinant yTBP was purified as described (Hoey et al., 1990), except that the heparin peak was further purified on a Mono S HR5/5 FPLC column (Pharmacia). Recombinant yTFIIA was purified as described (Ranish et al., 1992).

Supercoiling Reduction Assay

Plasmid chromatin was assembled and purified as described (Kwon et al., 1994). Reactions, total volume 12.5 μl, contained chromatin (2 ng of DNA), 1 U of topoisomerase I (Promega), 2.5 μl of 30% glycerol gradient buffer, 7 μl of buffer A minus KCl, 7 mM $MgCl_2$, 50–100 mM KOAc (final), 4 mM ATP where indicated, and 2 μl of holoenzyme Mono S fractions or 1 μl of SRB/SWI/SNF complex Mono Q fractions. Reactions were stopped after 90 min at 30°C by addition of 6 μl of stop buffer (3% SDS, 100 mM EDTA, 50 mM Tris–HCl [pH 8.0], 25% glycerol, 2 mg/ml proteinase K). Reactions were incubated for 90 min at 37°C and resolved on a 2% agarose gel (50 mM Tris–phosphate [pH 7.3], 1mM EDTA) for 40 hr, at 40 V. Gels were dried and exposed to film.

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REVIEW

Repression and activation by multiprotein complexes that alter chromatin structure

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The notion that chromatin structure might play an essential role in regulating gene expression was referred to in print as "the last refuge of scoundrels" as recently as 1986 (Brent 1986). Apparently, this view was widely held judging from the placement and time allotted to chromatin talks at transcription meetings. In the past decade, however, numerous structural, genetic, and biochemical studies have merged to present powerful arguments for the importance of chromatin structure in regulation. Chromatin structure on promoters has been shown to be dynamic, in that it can be altered during regulatory events in the absence of DNA replication (Schmid et al. 1992; for review, see Felsenfeld 1992). These data have been the subject of numerous recent reviews (Winston and Carlson 1992; Adams and Workman 1993; Becker 1994; Paranjape et al. 1994; Wolffe 1994; Orlando and Paro 1995; Roth 1995; Simon 1995). Here, we discuss the genetic and biochemical data that support the existence of multiprotein complexes whose primary function is to mediate changes in chromatin structure.

Over the past 50 years genetic studies in *Saccharomyces cerevisiae* and *Drosophila melanogaster* have identified numerous genes that are required for activation and repression. It now appears that some of these genes encode components of large complexes that interact directly with nucleosomes either to stabilize or destabilize nucleosome structure, leading to either repression or activation. The ongoing characterization of these complexes offers the hope that in the near future a direct mechanistic pathway leading from a promoter-bound activator or repressor to a regulated alteration in chromatin structure can be elucidated.

The characterization of these complexes is necessary to understand general transcriptional regulatory mechanisms and is likely to be particularly important in characterizing the mechanisms that regulate expression of genes during developmental processes. Many of the proteins that constitute the complexes discussed below were identified originally in genetic screens for mutations that affect processes such as appropriate segmentation in *Drosophila*, mating-type switching in yeast, and neoplasia in mammals (e.g., Stern et al. 1984; Kennison and Tamkun 1988; Haupt et al. 1991; van Lohuizen et al. 1991a). It has long been appreciated that changes in chromatin structure, as visualized by nuclease hypersensitive sites, accompany induction of tissue-specific genes during development and differentiation (Gross and Garrard 1988). The macromolecular complexes discussed below are likely to play an essential role in the maintenance of specifically altered chromatin states as cells differentiate during development.

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Regulated steps on chromatin templates

To understand how these complexes might function, it is first necessary to consider the steps in transcriptional regulation that might be altered by chromatin structure. Activation of a promoter in chromatin will require, at a minimum, binding by transcriptional activators, formation of the general transcription factors into an active preinitiation complex, and efficient initiation and elongation by RNA polymerase. Nucleosomes, the primary component of chromatin structure, have been shown to inhibit each of these steps in vitro (Fig. 1), and binding of histone H1 and formation of higher order structures almost certainly enhances these inhibitory effects (Lorch et al. 1987; Kamakaka et al. 1993; for review, see Felsenfeld 1992; Adams and Workman 1993). Many of these steps occur permissively on naked templates but are strongly inhibited in chromatin. For example, most activators will bind to their site with an affinity of at least 10^{-9} M on naked DNA. In contrast many activators bind with a K_d of 10^{-7} M or higher on nucleosomal templates (for review, see Adams and Workman 1993). The general factors TBP, TFIIA, and TFIIB are able to recognize most TATA sequences on naked DNA with a K_d of 10^{-9} to 10^{-10} M but are unable to bind at all to certain nucleosomal templates at concentrations as high as 10^{-6} M (Imbalzano et al. 1994). Similarly, transcriptional elongation occurs efficiently on most naked templates, but formation of the template into nucleosomes significantly inhibits the elongation rate of RNA polymerase II (Izban and Luse 1992), apparently because the nucleosome must "step around" the transcribing polymerase (Studitsky et al. 1994, 1995). Activators might function by recruiting complexes that decrease the inhibitory nature of chromatin structure at each of these steps, whereas

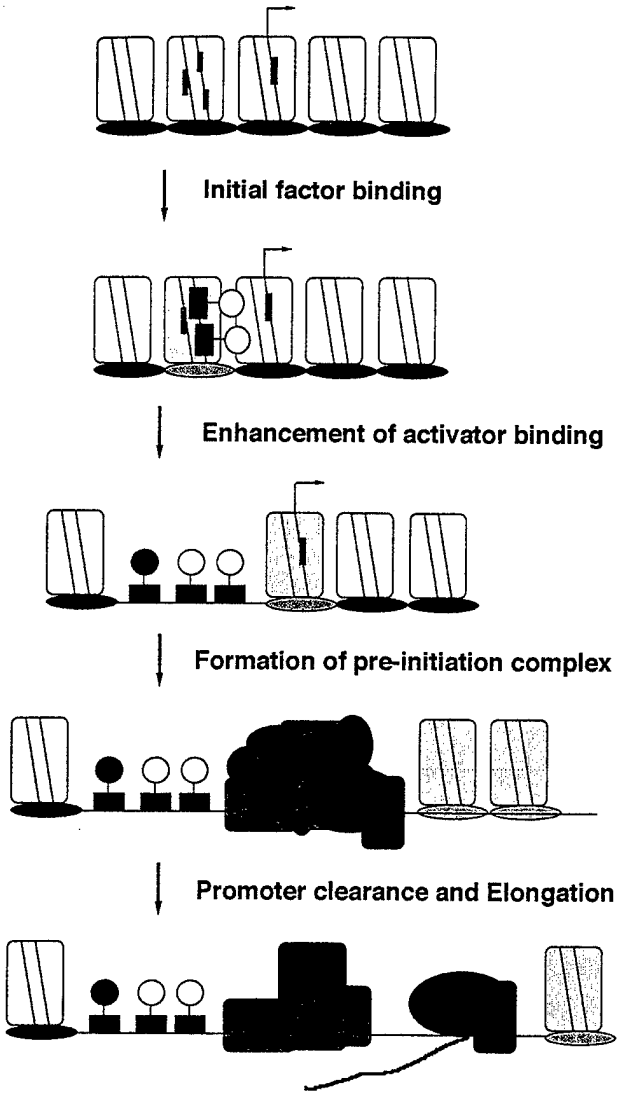

Figure 1. Steps in transcriptional activation that are inhibited by nucleosome formation. These steps are shown as occurring sequentially for clarity; it is likely that some of these steps occur in a concerted fashion in vivo.

repressors might recruit complexes that stabilize chromatin structure.

Activation on chromatin templates

By the early 1990s, genetic and biochemical studies on transcriptional activation suggested the existence of gene products that might be directly involved in modifying chromatin structure to achieve transcriptional activation. Mutations in the amino termini of histone H4 inhibited activation of the *GAL1* and *PHO5* promoters in *S. cerevisiae*, suggesting that factors might interact directly with these amino termini (Durrin et al. 1991). Biochemical analyses demonstrated that in mammalian, *Drosophila*, and yeast systems nucleosome formation could repress transcription, and that the addition of transcriptional activators could significantly alleviate such repression (Workman et al. 1988, 1991; Lorch et al. 1992; Kamakaka et al. 1993). In *S. cerevisiae*, function of activators on nucleosomal templates was found to require additional factors that might play a coactivating role, perhaps by altering chromatin structure (Lorch et al. 1992). These studies, and the genetic studies discussed below, prompted numerous laboratories to initiate a search for factors that might alter nucleosome structure to facilitate steps required for transcriptional activation.

The *SWI/SNF* genes

A group of genes in *S. cerevisiae* termed *SWI/SNF* have emerged as strong candidates to encode proteins that directly alter chromatin structure during transcriptional activation (for review, see Winston and Carlson 1992). The *SWI* genes were identified as being required for appropriate regulation of mating type switching (Stern et al. 1984; Breeden and Nasmyth 1987). A subset of these genes, *SWI1*, *SWI2*, and *SWI3*, were subsequently shown to be necessary for normal transcription of certain yeast genes, including *HO* (an endonuclease required for mating-type interconversion), *INO1* (an enzyme required for inositol metabolism), and *SUC2* (invertase; required for growth on sucrose and raffinose) and for function of some ectopically introduced activators such as the glucocorticoid receptor (Peterson and Herskowitz 1992; Yoshinaga et al. 1992). The SNF genes were identified as being necessary for *SUC2* transcription (Neigeborn and Carlson 1984), and the *SNF2*, *SNF5*, and *SNF6* genes were shown to be required for appropriate transcription from the long terminal repeat (LTR) of the Ty retrotransposon and for activation by fusion proteins containing the LexA DNA-binding domain and either the GAL4 or Bicoid activation domains (Happelet al. 1991; Laurent and Carlson 1992). In addition, SNF2 (which is identical to SWI2), SNF5, and SNF6 were all shown to function as activators when targeted to a promoter as LexA fusions (Laurent et al. 1991, 1993). These studies implied that SWI and SNF products play an important general role in transcriptional activation.

Genetic studies suggested that the SWI/SNF products might form a complex that actively disrupts chromatin structure. The *SIN* genes were identified as suppressors of the *swi*$^-$ phenotype, and *sin1* and *sin2* mutants are able to partially suppress mutants of the *SWI1*, *SWI2*, and *SWI3* genes (Sternberg et al. 1987; Kruger and Herskowitz 1991; Kruger et al. 1995). The *sin2-1* mutation was found to lie in the *HHT1* gene, which encodes histone H3. Subsequent work showed that a set of six different point mutations, three in histone H3 and three in histone H4, all displayed a *SIN* phenotype in that they would partially suppress the requirement for *SWI* genes in activation (Kruger et al. 1995). These mutations are all at residues believed to either contact DNA or to be involved in histone–histone contacts within the histone octamer, and thus all mutations might affect nucleosome stability. The *SIN1* gene was found to encode a

protein with homology to HMG-1, a chromatin structural component, and mutations in SIN1 affect chromosome segregation, supporting a role for this gene in chromatin formation (Kruger and Herskowitz 1991). Lowering the amount of histones H2A and H2B in the cell can also partially suppress *swi/snf* phenotypes (Hirschhorn et al. 1992). Thus, alterations in each of the four core histones and in a presumed chromatin structural protein all result in partial suppression of swi/snf mutations, suggesting that destabilization of chromatin structure can partially alleviate the requirement for SWI/SNF function. Although this is consistent with a role for the *SWI/SNF* genes in altering chromatin structure to allow function of the transcription machinery, it is possible that these genetic interactions do not reflect a direct functional interaction between SWI/SNF proteins and chromatin. It might be that mutation of chromatin components decreases the inhibitory effects of chromatin, thus making activation of transcription more permissive and alleviating a requirement for SWI/SNF in activation that is not related to altering chromatin structure.

Analyses of chromatin structure at the *SUC2* promoter in wild-type and *snf* mutant yeast strains support a direct role for SWI/SNF in disrupting chromatin structure (Hirschhorn et al. 1992; Matallana et al. 1992). When transcription of the *SUC2* promoter is induced the micrococcal nuclease sensitivity of the upstream promoter region is increased. Neither this alteration nor full transcriptional induction occurs in *snf5* or *swi2/snf2* mutants (Hirschhorn et al. 1992). Decreasing histone H2A and H2B levels not only partially suppresses the *swi/snf* phenotype of these mutants but also partially restores the disrupted (active) chromatin structure at *SUC2*. These data offer a strong correlation between the presence of SWI/SNF activity and a disruption of chromatin structure that accompanies transcriptional activation.

The genetic and structural data discussed above are equally compatible with two models for SWI/SNF function: SWI/SNF actively disrupts chromatin structure at promoters to allow increased access of the transcription machinery; or SWI/SNF proteins mediate the interaction between activators and general transcription factors to increase the ability of the general machinery to bind to chromatin. This latter model would postulate that the affinity of the transcription apparatus for sites in chromatin would be increased via SWI/SNF function without any direct effect on nucleosome stability; however, the increased affinity of the transcription apparatus for the promoter would allow it to displace nucleosomes. (Mutations that disrupt nucleosome structure would suppress the need for SWI/SNF function because of the decreased inhibitory effect of the altered nucleosomes.) This model has some support from the observation that the SWI/SNF proteins are associated with RNA polymerase holoenzyme (see below), which is the entity that is currently believed to be recruited to yeast promoters upon activation. Thus, activator contact with SWI/SNF proteins might be expected to increase affinity of holoenzyme for the promoter. This model is argued against, however, by the observation that SWI/SNF-dependent reorganization of chromatin structure at the *SUC2* promoter occurs on a crippled promoter that is decreased 20-fold in expression because of a TATA box mutation (Hirschhorn et al. 1992); this mutation should significantly inhibit binding of the transcription machinery, yet chromatin structure is still altered.

Further support for an active role for the SWI/SNF complex in disrupting chromatin structure comes from biochemical studies that demonstrate that the yeast SWI/SNF proteins form a multisubunit complex that alters nucleosome structure and that an apparent human homolog of the yeast SWI/SNF complex has similar properties. The initial evidence that SWI/SNF proteins form a large complex came from genetic studies; maximal activation by LexA fusions to SNF2 and SNF5 require the presence of other SNF and SWI products in the cell (Laurent et al. 1991). A complex of SWI/SNF proteins was subsequently identified by gradient sedimentation followed by immunostaining, and it was shown that the integrity of this complex was disrupted in strains that had null alleles for several *swi* and *snf* genes (Peterson et al. 1994). Subsequent purification of the complex indicated that it contains 11 subunits and includes the products of the originally identified genes (*SWI1*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6*) in addition to a newer gene product identified genetically (*SNF11*) (Cairns et al. 1994; Côté et al. 1994; Treich et al. 1995).

Purified yeast SWI/SNF has activities that are expected for a complex that is actively involved in disrupting chromatin structure during transcriptional activation. It was shown previously that SWI2/SNF2 has ATPase activity (Laurent et al. 1993), so function of the SWI/SNF complex was anticipated to be ATP-dependent: In fact, the purified complex was able to alter nucleosomal structure in an ATP-dependent manner, as measured by disruption of the DNase ladder that is characteristic of stable nucleosomes (Côté et al. 1994). It was also able to facilitate binding of derivatives of the transcriptional activator GAL4, again in an ATP-dependent manner. The SWI/SNF complex binds specifically to cruciform DNA structures, which might resemble the structure of nucleosomal DNA at the position where the DNA enters and leaves the nucleosome and induces positive supercoils in naked plasmid DNA (Quinn et al. 1996). Both of these activities on naked DNA might play a role in nucleosome destabilization, although both activities are not depedent on ATP, so it is unclear how they relate to the ATP-dependent processes of nucleosome disruption and factor loading.

The conservation of the ATP-dependent functions of SWI/SNF was demonstrated by the concomitant analysis of a presumed human homolog of the SWI/SNF complex. Two human genes with a high degree of similarity to yeast *SWI2/SNF2* were isolated and were termed *Brg1* and *hBrm* for their similarity to the *Drosophila* homolog of *SWI2/SNF2*, *Brahma* (Kennison and Tamkun 1988; Tamkun et al. 1992; Khavari et al. 1993; Muchardt and Yaniv 1993). Both hBRM and BRG1 proteins were shown to increase the ability of activators to function when

expressed by cotransfection in mammalian cells, indicating a functional similarity to SWI2/SNF2 (Khavari et al. 1993; Muchardt and Yaniv 1993; Chiba et al. 1994; Singh et al. 1995). An antiserum that recognized both BRG1 and hBRM was used to isolate two chromatographically distinct, highly enriched fractions termed hSWI/SNF A and hSWI/SNF B (Kwon et al. 1994). Both of these fractions were shown to disrupt nucleosome structure, as measured by alteration of DNase digestion patterns, and to facilitate the binding of GAL4 derivatives in an ATP-dependent manner (Imbalzano et al. 1994; Kwon et al. 1994). In addition, the hSWI/SNF fractions were shown to increase binding of TFIIA and TBP to the TATA sequence on nucleosomal DNA, a step that is potentially rate-determining during transcriptional activation. Subsequent studies with yeast RNA polymerase holoenzyme fractions that contain SWI/SNF also demonstrated an ATP-enhanced loading of TFIIA and TBP to the TATA box, showing that this property is conserved between humans and yeast as well (Wilson et al. 1996). In addition to these functional similarities between yeast and human SWI/SNF complexes, it appears that there will be similarities in the proteins that constitute each complex; a human gene termed *INI1* has been isolated that has extensive similarity to yeast SNF5, and the INI1 protein associates with BRG1 and cofractionates with hSWI/SNF (Kalpana et al. 1994).

These data suggested that an evolutionarily conserved function of the SWI/SNF complex is to disrupt nucleosome structure in an ATP-dependent manner to increase binding of transcription factors. Although these results, in combination with the genetic data on *SWI/SNF*, can be used to make a strong argument that nucleosome disruption is directly involved in activation, pieces of the puzzle are missing that would make the argument convincing: There are no data to show that SWI/SNF activity can be targeted to a promoter by activators, and there are no data that show a requirement for SWI/SNF activity in activation of transcription on nucleosomal templates in vitro, as this remains a technically challenging experiment.

Recent data have shed light on one of these issues—targeting. The ongoing characterization of factors that copurify with the yeast RNA polymerase II holoenzyme complex has shown that the SWI/SNF proteins cofractionate with this complex and can be coimmunopreciptated with the SRB proteins, the hallmark members of holoenzyme (Wilson et al. 1996). The SWI/SNF complex can be isolated from yeast without associated holoenzyme (Cairns et al. 1994; Côté et al. 1994), implying that the interaction with holoenzyme can be disrupted under certain chromatographic procedures and/or that some SWI/SNF is present in the cell in a form that is not associated with holoenzyme. Holoenzyme is a complex that contains numerous general transcription factors in addition to RNA polymerase II and the SRB proteins (for review, see Emili and Ingles 1995). Activators function efficiently in vitro in reactions that contain holoenzyme, and direct recruitment of holoenzyme to a promoter by activators has been proposed as a mechanism of activation (Kim et al. 1994; Koleske and Young 1994; Barberis et al. 1995). Holoenzyme contains a subset of proteins that interact with the carboxy-terminal domain (CTD) of RNA polymerase II-termed mediator (Kim et al. 1994), and the yeast SWI/SNF proteins also copurify with mediator (Wilson et al. 1996). Both the holoenzyme and mediator fractions have the expected ATP-dependent nucleosome disruption activity of SWI/SNF, and holoenzyme can assist TBP/TFIIA loading to a nucleosomal template in an ATP-enhanced manner. These data suggest that the yeast SWI/SNF proteins are associated with the complex that forms with the RNA polymerase CTD and that SWI/SNF activity might be targeted via association with holoenzyme. These biochemical observations were foreshadowed by the genetic observation that *sin* mutations, which suppress SWI/SNF function, also suppress the auxotroph phenotypes caused by CTD truncation (Peterson et al. 1991). It is unclear whether human SWI/SNF proteins are also associated with RNA polymerase holoenzyme, so the generality of these findings has not been established yet.

The data pertaining to SWI/SNF are compatible with a simple, testable model for transcriptional activation in yeast (Fig. 2). The model expands on previous proposals that recruitment of holoenzyme to a promoter is a critical aspect of activation (Barberis et al. 1995). If holoenzyme is recruited in a robust manner, nucleosomes are displaced as a result of the high affinity of holoenzyme binding, and SWI/SNF activity is not needed (Fig. 2). If holoenzyme recruitment is less robust, or there is a refractory chromatin configuration at the promoter, then the SWI/SNF activity associated with the holoenzyme disrupts nucleosomal structure to facilitate binding. This model explains why some promoters in which nucleosome disruption occurs with activation (e.g., *PHO5*) do not require SWI/SNF action, whereas other promoters such as *SUC2* do require SWI/SNF action. Such a model yields several testable hypotheses: SWI/SNF function should be targeted by some activators in vitro when holoenzyme fractions are used; weak activators should require SWI/SNF function in vivo in certain chromatin settings, whereas strong activators in the same setting should not; and mutations in *SWI/SNF* genes should exist that disrupt holoenzyme/SWI/SNF interaction, that only affect regulation of certain promoters, and that do not affect the general nucleosome disrupting abilities of SWI/SNF. A key point to consider with this model concerns the amount of SWI/SNF in yeast and whether all holoenzyme is associated with SWI/SNF. Estimates of amounts of SWI/SNF range from 100 to 200 complexes per cell as determined by fractionation (Cairns et al. 1994; Côté et al. 1994), which would provide enough to associate with ~10% of holoenzyme, to nearly stoichiometric with holoenzyme (1000–2000 copies per cell), as determined by comparative Western analysis (Wilson et al. 1996). If some holoenzyme is associated with SWI/SNF and some is not, it is critical to have a mechanism to target the SWI/SNF-containing holoenzyme to the proper promoter.

The model presented here (Fig. 2) simplifies the issue

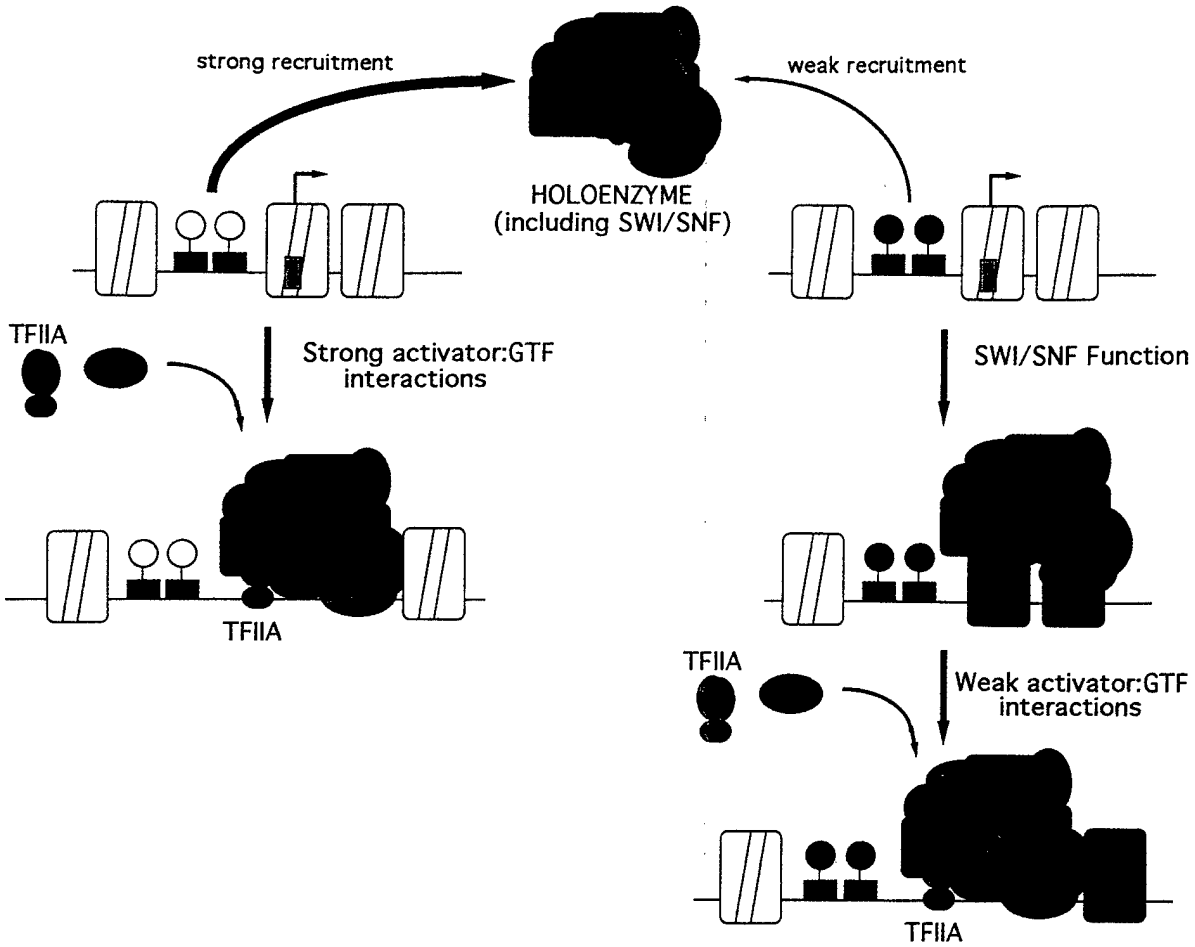

Figure 2. A model for function of SWI/SNF during activation in yeast. It is proposed that SWI/SNF is targeted to promoters via association with holoenzyme but that SWI/SNF function is only required when activator–holoenzyme contacts are insufficiently robust to allow holoenzyme to displace nucleosomes.

of how the activator initially finds its binding site by placing the activator on DNA prior to interaction with SWI/SNF or the general machinery. In some promoters (e.g., *PHO5*) the activator binds to linker DNA, whereas in other promoters activators must bind to a nucleosomal site. It is possible, if not likely, that SWI/SNF will mediate activator binding in vivo to nucleosomal sites, as it does in vitro, and that on certain promoters there might be a concerted reaction that loads activators and holoenzyme in a SWI/SNF-dependent manner. It is also possible that on certain promoters SWI/SNF acts independently of holoenzyme, whereas on others its action is targeted via association with holoenzyme.

ATP-dependent chromatin reorganizing activities in *Drosophila*

The biochemical studies of SWI/SNF were motivated by a desire to understand the mechanistic basis for *swi/snf* mutant phenotypes; a different approach that has uncovered ATP-dependent chromatin reorganizing activities has come from the development of in vitro chromatin reconstitution systems that seek to recapitulate changes in chromatin structure on regulated *Drosophila* genes. The *Drosophila* heat shock protein genes, particularly *HSP70*, have served as an important model system for characterizing changes in chromatin structure at promoters since 1980 (Wu 1980). The inactive *HSP70* gene is bound by a sequence-specific activator termed GAGA factor, the general transcription factor TFIID, and a transcriptionally engaged RNA polymerase that is paused at +25 (for review, see Lis and Wu 1993). DNase hypersensitive sites have been characterized in the promoter region, and their formation requires both the TATA sequence and the GAGA binding sites. In an effort to understand the genesis of these hypersensitive sites, an S190 extract from *Drosophila* embryos was developed that could create appropriately spaced nucleosomes on plasmid templates in vitro and that could be used to study the effects of transcription factor binding on chromatin structure (Becker and Wu 1992).

Binding of GAGA factor to HSP70 templates that have been assembled into chromatin with these extracts generates a disruption in the micrococcal nuclease digestion pattern at the promoter and also results in DNase hypersensitivity that is similar, but not identical, to the hy-

persensitivity that occurs in vivo (Tsukiyama et al. 1994). These changes in nuclease accessibility require hydrolyzable ATP. Subsequent studies have shown that a separate activator, heat shock factor, can also create alterations in nuclease sensitivity on chromatin assembled by these extracts, and that restriction enzyme access to sites on these templates is increased in an ATP-dependent manner (Tsukiyama and Wu 1995; Varga-Weisz et al. 1995; Wall et al. 1995). The increased access of restriction enzymes and the increased perturbation of structure by factor binding has been proposed to result from an increased mobility of nucleosomes; nucleosome spacing on assembled templates in these extracts can be altered by changing salt concentration under appropriate conditions, and this salt-induced change also occurs in an ATP-dependent manner (Varga-Weisz et al. 1995). In parallel studies, a *Drosophila* extract prepared under somewhat different conditions could assemble ordered nucleosomal arrays, and the fidelity of these arrays was altered by binding of the activator GAL4, also in an ATP-dependent manner (Pazin et al. 1994).

These data indicate that *Drosophila* embryos contain proteins that can alter nucleosome mobility, increase restriction enzyme access, and permit activator-dependent alterations in chromatin structure, all in an ATP-dependent manner. It is not clear whether these capabilities are all the result of one complex of proteins or whether several different complexes are involved.

NURF

In an elegant study, Tsukiyama, Wu, and colleagues have purified an activity from *Drosophila* embryo extracts, termed NURF (nucleosome remodeling factor), that is able to facilitate perturbation of chromatin structure by the GAGA factor and increase restriction enzyme access in an ATP-dependent manner (Tsukiyama and Wu 1995). The final fraction from this purification contains four predominant polypeptides and was capable of promoting GAGA factor and ATP-dependent disruption of micrococcal patterns at low ratios of NURF to nucleosomes (very approximately, 10 nucleosomes per NURF complex). NURF also displayed activities similar to SWI/SNF; it could alter the 10-bp DNase digestion ladder on a mononucleosome in an ATP-dependent manner and facilitate binding of GAGA factor to a mononucleosome in an ATP-dependent manner. These data led to a model in which NURF alters nucleosomal DNA structure in an ATP-dependent manner, which then facilitates GAGA factor binding (Tsukiyama and Wu 1995). These alterations might conceivably result in an increased mobility of nucleosomes on large templates (Varga-Weisz et al. 1995), with concomitant changes in micrococcal sensitivity and restriction enzyme access, although further study is needed to understand how the increased restriction enzyme access and nuclease access are related to each other and to nucleosome mobility.

In a striking convergence of the biochemical studies of *Drosophila* extracts and the genetic studies of SWI/SNF-related factors, one of the subunits of NURF has been identified as the product of the *ISWI* (imitation switch) gene (Tsukiyama et al. 1995). The *ISWI* gene was identified in a low-stringency Southern screen for *Drosophila* factors with sequence similarity to yeast *SWI2/SNF2* (Elfring et al. 1994). The *Drosophila* gene with highest homology to *SWI2/SNF2* is *Brahma* (*Brm*); however, *ISWI* has significant identity to *SWI2/SNF2* (and *BRM* and the human genes *hBRM* and *BRG1*) over a 460-amino-acid ATPase domain. A human gene, *hSNF2L* (Okabe et al. 1992), is 75% identical to *ISWI* over its entire length and therefore appears to be the human homolog of *ISWI*. A putative yeast homolog (termed *YB95*) has also been identified, indicating that *ISWI* is extensively conserved (Tsukiyama et al. 1995).

NURF and SWI/SNF as facilitators of activation

It is attractive to postulate that the NURF and SWI/SNF-related complexes represent two distinct classes of ATP-dependent nucleosome reorganizing activities that both function during activation of gene expression, with the separate complexes being used in separate circumstances. Each complex has been shown to alter nucleosome structure in a manner that facilitates transcription factor interaction in vitro. The extensive conservation of the ATPase of each complex (SWI2/SNF2 and ISWI) from yeast through humans suggests an important role for each complex in vivo. The nucleosome remodeling activities of each complex might be used during the various stages of transcription that are inhibited by nucleosomes (see Fig. 1) but also might be used in chromatin assembly or in recombination reactions. The requirement of yeast *SWI/SNF* genes for function of several activators (Laurent and Carlson 1992; Peterson and Herskowitz 1992; Yoshinaga et al. 1992), the requirement of brm for appropriate maintenance of homeotic gene expression in *Drosophila* (Kennison and Tamkun 1988; Tamkun et al. 1992), and the association of yeast SWI/SNF with RNA polymerase holoenzyme all offer support for a role of the SWI/SNF complex in transcriptional activation (Wilson et al. 1996). There are no data on the phenotype of *ISWI* mutations in *Drosophila*; it will be intriguing to test the hypothesis that NURF is required on genes that utilize GAGA factor in vivo.

Although the biochemical activities of each complex argue for a direct role in the transcription activation process, it is important to emphasize that neither activity has been shown explicitly to play a role in transcriptional activation in vitro. The availability of model transcription systems (e.g., GAL4-driven promoters) and highly fractionated complexes should allow an assessment of the role of these complexes in activation; however, a more attractive in vitro experiment would be to use a natural promoter that requires either SWI/SNF or NURF activity in vivo to characterize the role that these complexes might play. Unfortunately, there is a major stumbling block to such a strategy: There are no mammalian or *Drosophila* genes whose regulation has been demonstrated to require SWI/SNF or NURF complexes in vivo, and there are no established in vitro chromatin

systems to study the yeast genes, such as *SUC2* or *HO*, that require SWI/SNF function. Thus, although an important role for these complexes in activation is likely, there is a need for significant further experimentation for a rigorous argument to be made.

It is conceivable that the SWI/SNF and NURF complexes play redundant roles in vivo. Both display similar abilities to disrupt nucleosomal structure and facilitate factor loading (Côté et al. 1994; Imbalzano et al. 1994; Kwon et al. 1994; Tsukiyama and Wu 1995). There are apparent differences in the ATPase activities of the two complexes, however, as the ATPase activity of NURF is modestly stimulated by nucleosomal DNA and is not significantly stimulated by naked DNA (Tsukiyama and Wu 1995), whereas the SWI/SNF ATPase activity can be stimulated by naked DNA (Laurent et al. 1993; Côté et al. 1994; Kwon et al. 1994). In addition, the ISWI ATPase domain will not substitute for the SWI2/SNF2 ATPase domain in yeast gene replacement studies, whereas the BRM ATPase domain will (Elfring et al. 1994). These data, in conjunction with the greater size and complexity of SWI/SNF-related complexes (2000 kD; 11 or more peptides) versus the NURF complex (500 kD, 4 peptides), suggest that the two complexes play distinct roles.

Sorting out the relative roles and functions of these complexes will be complicated further by the number of SWI/SNF-related complexes in mammals. There are at least two chromatographically distinct complexes in HeLa cells, and the two mammalian homologs of *SWI2/SNF2*, *hBRM* and *BRG1*, each have two splice variants (Khavari et al. 1993; Muchardt and Yaniv 1993; Chiba et al. 1994). The different splice variants of Brg1 have been shown to have differential effects on cell growth. Both *BRG1* and *hBRM* can inhibit cell growth, and both interact with the hypophosphorylated form of the retinoblastoma protein (Rb); the two differentially spliced forms of Brg1 show different abilities to interact with Rb and to slow cell growth (Dunaief et al. 1994; Strober et al. 1996). These data raise the possibility of multiple forms of mammalian SWI/SNF-related complexes, each containing different members of the mammalian homologs of the *SWI2/SNF2* genes, and each potentially having different abilities to function in chromatin.

Chromatin-specific activators

If the recruitment of chromatin remodeling complexes is an important aspect of activation, and it is not obligatory that these activities be recruited with general transcription factors such as holoenzyme, then one might expect that certain activators would function solely by recruiting remodeling complexes. This hypothetical class of activators would be expected to function only on chromatin templates. GAGA factor in *Drosophila* and LEF-1 in mammals might be members of such a class, as they seem to function in a chromatin-specific manner.

GAGA factor binding sites are required for establishment of proper chromatin structure on the promoters of the *HSP26* and *HSP70* genes (for review, see Lis and Wu 1993). Purified GAGA factor has been shown to remodel chromatin structure in conjunction with NURF (Tsukiyama et al. 1994; Tsukiyama and Wu 1995) and has been shown to alleviate repression of transcription in vitro on templates that have been repressed by histone H1 (Crosten et al. 1991). The factor does not stimulate transcription in vitro on naked DNA templates. In mammalian systems, LEF-1 has been shown to bend DNA and has been proposed to play an important architectural role in establishing proper enhancer structure (Geise et al. 1992) but does not stimulate transcription on naked DNA templates. LEF-1 is able, however, to stimulate transcription significantly from the HIV enhancer in vitro when that enhancer has been assembled into a spaced nucleosomal array (Sheridan et al. 1995). It will be important to determine whether specific domains of factors such as these are required for function on chromatin and whether these domains are demonstrably different from characterized transcriptional activation domains. If remodeling of chromatin is a critical aspect of activation, then it would be anticipated that there would be chromatin-specific activation domains that might direct remodeling.

Chromatin structure and repression of gene expression

The complexity of genes believed to be involved in activation on chromatin templates is daunting, and there appears to be at least a similar degree of complexity in genes that might play a role in establishing and maintaining repressive chromatin structures. The importance of maintaining stable repression of gene expression is most apparent in multicellular organisms, where it is essential that developmental regulatory genes be active only in specific cell types. In fact, there are extensive genetic studies dating from the discovery of the *Polycomb* gene in *Drosophila* in 1947 implying that there is a large network of genes that are required to maintain repression of transcription during development (referred to as the *Polycomb-group*, or *Pc-G*, in *Drosophila*). These genes were among the earliest identified in screens for homeotic transformations that led to abnormal expression of sex combs on the fly. The general importance of these gene products is indicated by the isolation of human homologs of *Pc-G* genes as proto-oncogenes (e.g., *Bmi-1*, which collaborates with Myc in inducing tumors; Haupt et al. 1991; van Lohuizen et al. 1991b). It is currently believed that these gene products function by establishing a stable, repressed chromatin structure over promoters (see below).

Pc-G genes, in contrast to *SWI2/SNF2* and *ISWI*, do not appear to be conserved in yeast. A role for chromatin structure in gene repression is well characterized in yeast, however, and the mechanisms might be general. The complexity of the genes potentially involved in repression in chromatin precludes a thorough review of this area here; recent reviews have covered silencing, *SSN6/TUP1*, and the *Pc-G* in greater detail (Orlando and Paro 1995; Roth 1995; Simon 1995). Topics such as position–effect variegation are also relevant but beyond the scope of this review. The remainder of this review will

focus on data indicating that repressive complexes are targeted to specific genes, potentially "coating" chromatin via contact with the amino termini of nucleosomes, and it will discuss the possibility that these repressive complexes function in direct competition with the remodeling complexes believed to be involved in activation.

Silencing at telomeres and at *HML* and *HMR*

A very potent repression of transcription in *S. cerevisiae*, silencing, occurs near telomeres and at normally unexpressed copies of yeast mating-type genes *HML* and *HMR*. This type of repression is perhaps related to the condensation of chromatin that occurs in heterochromatin in multicellular organisms, as the *HM* loci and telomeres share with heterochromatin the properties of late replication, "spreading" of silencing, and variegation (for references, see Hecht et al. 1995). It appears that transcriptional repression at telomeres and at the silent mating type loci *HML* and *HMR* in yeast is mediated by a complex that contains (at minimum) the nucleosome and several SIR (silent information regulator) gene products. This repression also involves other proteins, such as the sequence-specific binding protein RAP1, which binds promoters that are not silenced and also to silenced regions, and the origin recognition complex (ORC), which binds specifically to DNA replication origins (Rine and Herskowitz 1987; Shore and Nasmyth 1987; Aparicio et al. 1991; Laurenson and Rine 1992; Chien et al. 1993; Renauld et al. 1993; Morettti et al. 1994; Hecht et al. 1995; for review, see Newlon 1993; Shore 1994). A direct involvement of histones in silencing was suggested by studies showing that deletion of the amino termini of either histone H3 or histone H4 derepressed the *HM* loci and genes located near telomeres (Kayne et al. 1988; for review, see Hecht et al. 1995). Genetic studies have also implicated several other genes in this repression, and current data suggest that SIR3 and SIR4 might interact directly with histone amino termini to nucleate a repressive structure.

The *SIR* genes were isolated via their requirement for efficient repression at the *HM* loci (for review, see Rine and Herskowitz 1987), and the *SIR2*, *SIR3*, and *SIR4* genes were subsequently found to be necessary for repression at telomeres (Aparicio et al. 1991). Telomeres create a heritably repressed state that can extend for varying distances along the chromosome from the telomere to adjacent genes, and that varies in extent of spreading in genetically identical cells (producing a variegated phenotype) (Gottschling et al. 1990). Gene replacement strategies have been used to show that if a distal gene is repressed by a telomere in a given cell, a proximal gene in the same cell is also repressed, indicating that repression spreads from the telomere outward (Renauld et al. 1993). SIR3 has been directly implicated in this spreading by studies showing that overexpression of SIR3 leads to increased spreading (Renauld et al. 1993). As in silencing of the *HM* loci, potential targets of the *SIR3* gene include the amino termini of histones H3 and H4: Deletions and point mutations in these amino termini cause derepression of *HM* loci and telomeres, and the regions delineated by these mutations differ from the regions of the amino termini that are required for transcriptional activation (for an overview, see Hecht et al. 1995; Roth 1995).

Recent experiments offer support for a direct interaction between SIR3 and SIR4 and the amino termini of H3 and H4 (Hecht et al. 1995). Glutathione *S*-transferase (GST) pull-down experiments were used to show that SIR3 and SIR4 could interact with the amino termini of histones H3 and H4 but not the amino termini of histones H2A and H2B, which are similarly charged. There was a very good, but not absolute, correlation between the effects of histone mutations on interaction in this assay with the effects on derepression in vivo. Finally, SIR3 localizes to specific perinuclear regions, similar to those where the telomere-specific binding protein RAP1 localizes (Palladino et al. 1993), and histone H3 and H4 deletions were shown to cause delocalization of SIR3 in vivo (Hecht et al. 1995).

Although these studies offer strong support for the hypothesis that SIR3 plays an essential role in directing a repressive structure that interacts with histone amino termini (see Fig. 3), there are not yet direct functional studies to corroborate this hypothesis. Silencing is known to affect the general accessibility of chromatin in vivo, as shown by decreased restriction enzyme and DAM methylase access to DNA in silenced regions (Gottschling 1992; Loo and Rine 1994). By analogy with the increase in restriction enzyme accessibility afforded by NURF and related complexes in vitro, a more complete understanding of the role that SIR3 plays in establishing a repressed structure would be aided by the establishment of in vitro systems where SIR3-containing complexes were shown to decrease access of proteins to DNA, either over the already refractory ground state of nucleosomal templates assembled in vitro or against action of complexes such as SWI/SNF.

A repressive complex that contains SIR3 is expected to contain numerous components. Two-hybrid studies have been used to show that SIR3 and SIR4 will interact with each other and with the sequence-specific factor RAP1 (Moretti et al. 1994). Genetic evidence indicates a critical role for the multisubunit ORC in establishing silencing at *HM* loci (for review, see Newlon 1993), and it has been suggested that ORC might also play a role at telomeres (Palladino and Gasser 1994). Other proteins, such as ABF1 and RIF1 are involved at silencing at *HM* loci, and there might be further factors needed at telomeres that remain uncharacterized. It is not clear which of these proteins are associated in a stable complex (SIR2, SIR3, SIR4, and nucleosomes?), which are involved in targeting the complex (SIR1, RAP1?), and whether the involvement of ORC is caused by a necessary role for replication in establishing the silenced state, and/or whether ORC plays a more direct role in establishing a refractory chromatin structure (see Chien et al. 1993; Fox et al. 1995).

Repression in silenced regions can be modulated by

activators. Overexpression of the PPR1 activator caused derepression of the *URA3* gene when the gene was located next to a telomere, and repression of a telomeric GAL4-driven reporter was derepressed by GAL4 expression in an activation domain-dependent manner (Aparicio and Gottschling 1994). This derepression could not occur in cells that were arrested in G_0, G_1, or early S but could occur in cells that were blocked in G_2/M. This observation suggests that the repressive structure might have to reform in G_2/M, allowing a window where formation of the repressive complex was in competition with activator function, and that activator function can block formation of a gene into a silenced state. Such a model is consistent with studies in multicellular organisms that imply that activator and Pc-G binding are mutually exclusive (see below). If activators do target remodeling complexes, then remodeling complexes and silencing complexes might be placed in direct competition on chromatin, with activator function playing a critical role in determining the resultant chromatin configuration.

SSN6/TUP1

In contrast to silencing, which creates a heritable repressed state that is rarely derepressed in a natural setting, the SSN6 and TUP1 proteins play a role in repressing genes that are derepressed under appropriate growth conditions, such as the **a**-specific genes (repressed in α cells), the *SUC2* gene and the *GAL1/GAL10* genes (for review, see Johnson 1995; Roth 1995). Repression by these proteins has been strongly correlated with the establishment of an ordered nucleosomal array over affected promoters. Neither SSN6 nor TUP1 is a sequence-specific DNA-binding protein. Rather, they are targeted to specific promoters via an interaction with sequence-specific factors such as α2/MCM1 (Johnson 1995). Targeting of either SSN6 or TUP1 to a promoter via fusion with the LexA DNA-binding domain also causes repression (Tzamarias and Struhl 1994). The LexA–SSN6 protein requires TUP1 expression to repress, whereas, intriguingly, LexA–TUP1 does not have an absolute requirement for SSN6, suggesting that TUP1 may be the more directly acting of the two proteins. These proteins fractionate from yeast cells in a large complex (1200 kD; Williams et al. 1991), raising the possibility that they might nucleate a repressive complex on chromatin.

The chromatin structure of numerous genes that are regulated by SSN6/TUP1 has been examined, and in all cases there is an ordered chromatin structure under repressive conditions (Roth 1995). Derepression of several **a**-specific genes, the *SUC2* gene and the *GAL1/GAL10* genes is accompanied by an alteration in chromatin structure (Roth et al. 1990; Shimizu et al. 1991; Axelrod et al. 1993; Cavalli and Thoma 1993; Cooper et al. 1994; Hirschhorn et al. 1995). The establishment of an ordered nucleosomal array on the **a**-specific genes requires SSN6 and TUP1 (Cooper et al. 1994), whereas SSN6 is necessary to establish the repressed structure on the *SUC2* gene (Matallana et al. 1992). Establishment of a repressed state requires histone amino termini, raising the possibility that repressive structures form that directly involve nucleosomes (Roth et al. 1992). The same conceptual difficulty discussed above for activation of transcription in chromatin pertains here; it is unclear whether there is a correlation between an ordered array and repressed genes because SSN6 directs a nucleosomal structure that represses steps required for transcription (Fig. 1), or because SSN6/TUP1 inhibits function of the general transcription machinery, and the resultant lack of transcription allows nucleosomes to form an ordered structure. One prediction of the latter model—in which a repressed structure does not require SSN6 or TUP1 for formation—would be that a promoter crippled by mutation of TATA sequences and/or upstream activating sequences would be packaged in ordered nucleosomes even in the absence of SSN6/TUP1 function. This is not true; mutation of either SSN6 or TUP1 alters nucleosome structure even on a crippled promoter, supporting a direct role for these proteins in creating a repressive chromatin state (Cooper et al. 1994).

Function of activators certainly involves direct interaction of the activators with the general transcription machinery in addition to effects on chromatin structure; it seems likely that global repressors such as SSN6/TUP1 also function via both mechanisms. SSN6/TUP1 are able to repress transcription in vitro in the absence of chromatin assembly (Herschbach et al. 1994). The extent of repression (4-fold) is much lower than seen in vivo (>100-fold), which might reflect either the difficulties of recapitulating full effects in an in vitro system or might indicate the extent to which SSN6/TUP1 functions via chromatin structure. In regulated settings, it would seem most efficient to use multiple mechanisms to achieve repression, as is likely to be the case for regulated activation.

Pc-G

The emerging roles for chromatin structure in repression of yeast genes serve as a paradigm for understanding how the *Pc-G* genes might function to maintain repressed states in multicellular organisms. The *Pc-G* genes were found in screens for effects on development in *Drosophila*, and this conserved set of genes is understood in most detail in this organism. There are 13 genetically identified members of this group, with perhaps at least an equal number remaining undiscovered (for review, see Simon 1995). Biochemical studies and in situ localization studies are interpreted most simply as indicating that the Pc-G gene products form several large complexes. The proteins encoded by Pc, polycomb-like (Pcl) and polyhomeotic (Ph), all localize to an identical set of ~100 bands in polytene chromosomes (Zink and Paro 1989; DeCamillis et al. 1992; Franke et al. 1992; Lonie et al. 1994). This remarkable colocalization, with data indicating that Pc and Ph coimmunoprecipitate and fractionate in an apparent complex >2 MD in size, suggests that these proteins are found in a single complex (Franke et al. 1992). The products of the *posterior sex combs* (*Psc*)

gene and the *suppressor 2 of zeste* [*Su(z)2*] gene colocalize with Pc, Ph, and Pcl at a subset of sites. Su(z)2 and Psc can also localize independently of each other and are also found together at sites where Pc, Pcl, and Ph are not found (Rastelli et al. 1993). Thus, Psc does not always colocalize with Pc/ph/Pcl, and it would seem most likely that Psc can sometimes be found in a different complex [sometimes containing Su(z)2?] in vivo. Fractionation of these complexes will be required to test these speculations concerning the multiplicity of Pc-G complexes.

The repressive effects of the Pc-G have been studied most thoroughly with the adjacent *Bithorax complex* (*BX-C*) and *Antennapedia complex* (*ANT-C*) gene clusters. These clusters each encode several homeotic genes required for appropriate development, and many of the *Pc-G* genes were initially identified as mutations that altered expression of these genes, resulting in misexpression of sex combs (Lewis 1978; Struhl 1981; Ingham 1983). Regulatory elements of the *Antp* and *Scr* genes of the *ANT-C* and the *Ubx*, *abdA*, and *abdB* genes of *BX-C* have been shown to confer regulation by the *Pc-G* on a linked *lacZ* reporter when introduced into *Drosophila* by P-element-mediated transformation (Zink et al. 1991; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Gindhart and Kaufman 1995). These sequences (termed PREs) are therefore believed to target Pc-G induced repression, although the mechanism by which these sequences are recognized by the Pc-G proteins is not clear, as no Pc-G protein has been shown to bind specifically to DNA. The most well-delineated completely functional PRE is a 5.6-kb fragment of the Ubx regulatory region (Chiang et al. 1995), and a 1.6-kb fragment from this region bestowed partial PRE function (Chan et al. 1994), so it does appear that there are simple, short sequence elements that can target Pc-G repression.

Once targeted, the Pc-G maintains repression of homeotic genes, apparently through the creation of a refractory chromatin structure. In considering Pc-G function, it is important to recall that the Pc-G must maintain repression of loci over numerous cell divisions: expression of homeotic genes is appropriately established in Pc-G mutant embryos, but these mutants lose repression as the fly develops, resulting in misexpression of homeotic genes later in development (for review, see Paro 1995; Simon 1995). Thus, the Pc-G proteins must perform a function similar to the proteins involved in silencing in yeast, and similar "coating" models have been proposed for both forms of repression (Paro 1990). Studies employing UV-cross-linking followed by immunoprecipitation have shown that the Pc protein is physically associated with sequences corresponding to entirety of the *Ubx* and *AbdA* genes (~300 kb), whereas it is not associated with the adjacent *AbdB* gene (Orlando and Paro 1993). Many of these sequences do not contain high-affinity Pc-G-binding sites when separated from the *BX-C* (Chiang et al. 1995); high affinity binding in this case is measured by the ability to confer repression on a linked *lacZ* gene and by the ability to target Pc as visualized by indirect immunofluorescence. Four fragments from this region, each 5–10 kb in length, do confer high-affinity binding by Pc. These data are consistent with a model similar to that proposed for silencing in yeast: The high affinity binding sites target and nucleate formation of a Pc-G complex, and the complex then spreads to adjacent regions, creating a stably repressed chromatin structure.

Establishment of a repressed chromatin state with similar properties to heterochromatin and/or silenced domains is an attractive hypothesis for Pc-G function, but is there direct evidence for formation of an altered chromatin structure? Suggestive evidence abounds: The Pc protein contains a region termed the "chromodomain" that is conserved with the heterochromatin-specific protein HP1 (Paro and Hogness 1991); the *Drosophila* homolog of the *SWI2/SNF2* gene, *BRM*, was isolated as a suppressor of Pc mutations (Kennison and Tamkun 1988; Tamkun et al. 1992); the ability of sequence-specific DNA-binding proteins to interact with *Pc-G*-repressed genes is inhibited in a manner similar to inhibition by stable nucleosomes (Zink and Paro 1995; McCall and Bender 1996). Perhaps the most compelling evidence that the Pc-G establishes a repressive chromatin structure is that some *Pc-G* genes can modify position–effect variegation (PEV) and some genes required for PEV can interact genetically with the *Pc-G* (Grigliatti 1991). PEV is presumed to result from spreading of heterochromatin, and similar variegated phenotypes occur through silencing mechanisms in yeast, so these results link Pc-G both to heterochromatin formation and to a mechanistic similarity with silencing. These observations all point to a Pc-G induced, refractory chromatin structure, but a physical association between Pc-G proteins and nucleosomes has not been detected yet, and there are no functional in vitro systems where Pc-G repression can be recapitulated and characterized.

Several results indicate that the repressed structure established by the Pc-G is not completely impervious to factor interaction and that the repression can be regulated by upstream activator proteins. Insertion of a GAL4-regulated reporter into the *Ubx* regulatory region places GAL4-driven activation under control of the Pc-G; however, T7 RNA polymerase transcription of genes in the same region is not repressed by the Pc-G (McCall and Bender 1996). Thus, not all sequence-specific DNA–protein interactions are blocked by Pc-G function. In a related experiment, a reporter containing both a PRE and GAL4 sites was integrated at sites that do not normally bind Pc-G proteins. GAL4 binding and Pc binding under these conditions appeared to be mutually exclusive when visualized by in situ hybridization (Zink and Paro 1995). Moreover, increased expression of GAL4 decreased Pc binding, suggesting a competition between the activator and the Pc-G in this setting. A direct link between activators and Pc-G repression is further supported by experiments where Pc-G members are targeted to promoters via heterologous DNA-binding domains. GAL4–Pc-G fusions repress different promoters to different extents in flies (Müller 1995), and LexA–Pc-G fusions are able to repress expression in mammalian cells

in a manner that varies according to the activation domain of the affected activator (Bunker and Kingston 1994). Thus, as discussed above for silencing in yeast, it appears that activators might block the function of the Pc-G proteins, either directly or via their potential to recruit remodeling complexes that might block formation of a repressed chromatin state.

Nucleosome mobility as a target for regulatory complexes

All of the above studies are consistent with a model that invokes nucleosome mobility as a key controlling mechanism in gene regulation (Fig. 3). Nucleosomes are frequently thought of as being static structures in which the histone core maintains stable contact with a particular 145-bp sequence. In fact, isolated nucleosomes have been clearly demonstrated to be mobile, meaning that they can readily change translational position on a piece of DNA, and this mobility can be affected by changes in solution conditions (Meersseman et al. 1992). Increased nucleosome mobility might result from the sliding of nucleosomes along DNA, or from the direct transfer of the nucleosome from one segment of DNA to an adjacent segment in a mechanism similar to that proposed for "stepping around" a transcribing RNA polymerase (Studitsky et al. 1994). An ATP-dependent activity in *Drosophila* extracts can increase mobility, and decreased mobility represses RNA polymerase III transcription, leading to previous proposals that regulated mobility might play a critical role in activation and repression (Ura et al. 1995; Varga-Weisz et al. 1995). Increased mobility of nucleosomes would increase the probability that a binding site for an activator or for a general transcription factor would become accessible, whereas decreased mobility would decrease that probability. Once a factor is bound, a nucleosome with increased mobility might be displaced more readily, either by sliding to an adjacent sequence or by transferring to a different stretch of DNA or to histone-binding proteins (see below). ATP-dependent remodeling activities such as NURF and SWI/SNF have been shown to increase accessibility of nucleosomal DNA to nucleases and sequence-specific binding factors; an increased mobility of nucleosomes is one potential cause for these observations. A stable structure formed between the SIR proteins and histone amino termini in yeast, as proposed for silencing, would very likely inhibit nucleosome mobility by adding the extra constraint of stable histone–regulatory protein interactions to that of histone–DNA interactions.

By this model, repressors could function both by directing formation of a structure that decreased nucleosome mobility and by blocking the recruitment of factors that increased mobility, whereas activators would function in the opposite manner. The likelihood that repressors and activators can both affect both types of remodeling complexes is favored by the a priori assumption that a dual capability for a regulatory protein would provide for more potent action. An ability to affect both types of complexes would explain why activators can help displace Pc-G complexes and can also prevent silencing (Aparicio and Gottschling 1994; Zink and Paro 1995). There is no necessity, and in fact no expectation, that activation and repression mechanisms that use these complexes will function solely through effects on chromatin structure; direct effects on general transcription factor function are also likely.

Structural considerations

To test the above hypothesis, and to test most any hypothesis concerning transcriptional regulation on chromatin, a deeper understanding of the numerous aspects of chromatin structure is needed. Below we briefly mention several aspects of chromatin structure that are likely to be critical to the function of regulatory complexes. These topics are all worthy of lengthy review; the brief descriptions below are meant to point out the complexity of the issues involved.

Histone amino termini

The amino termini of histones are not ordered in the available crystal structures of nucleosomes (Richmond et al. 1984; Arents et al. 1991; Arents and Moudrianakis 1993) and appear to stick out of the nucleosome core. This allows their positively charged surfaces to interact with DNA that is either bound to the core nucleosome,

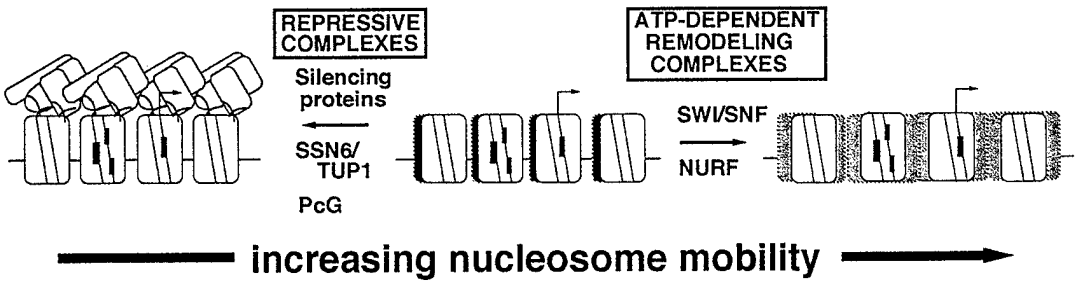

Figure 3. Nucleosome mobility as a key determinant of promoter activity. Multiprotein complexes might either increase or decrease nucleosome mobility to facilitate or inhibit transcription factor interaction (based on Meerseman et al. 1992; Hecht et al. 1995; Ura et al. 1995; Varga-Weisz et al. 1995).

is in linker regions, or is on an adjacent nucleosome. DNA cross-linking studies have been used in several instances to assign specific contacts for the various amino termini of the core histones (Pruss et al. 1995). Specific mutations in amino termini have effects on activation, silencing, and repression by SSN6/TUP1 (see above). Amino termini can be acetylated on lysine residues, causing a decrease in their positive surface charge, and presumably affecting their binding to DNA. Hypoacetylation has been strongly correlated with inactive genes in yeast and in multicellular organisms (Braunstein et al. 1993; Jeppesen and Turner 1993), whereas hyperacetylation is associated with active genes (although in this latter case the correlation is not complete) (Hebbes et al. 1988; Bresnick et al. 1991; Bone et al. 1994).

These data suggest an important role for amino termini in modulating nucleosome–DNA interactions and thereby modulating the repressive effects that nucleosomes have on transcription factor interactions. In fact, deletion of amino termini has been shown to increase binding of TFIIIA and GAL4 to nucleosome cores, and hyperacetylation has also been reported to increase binding of TFIIIA (Lee et al. 1993; Vettese-Dadey et al. 1994). As discussed above, the amino terminus of histone H4 has been shown to contact SIR3, perhaps nucleating formation of a repressive complex. Biophysical analysis demonstrates an important role for amino termini in the structuring of nucleosomal arrays, as judged from the effects of acetylation or removal of amino termini on the supercoiling of circular templates and sedimentation (Norton et al. 1989; Fletcher and Hansen 1995). It is clear from this body of work that further structural and biophysical studies on the function of amino-terminal tails are required for a full understanding of how factors interact with nucleosomes and how regulatory complexes might alter chromatin structure.

Nucleosome-associated proteins

Acidic proteins, such as nucleoplasmin in frogs and NAP-1 in yeast, are capable of binding histones and thereby facilitating removal of nucleosomes from DNA. These proteins have been shown to increase binding of factors such as GAL4, USF, and Sp1 and will increase dissociation of histones from a nucleosome that has been bound by an activator (Chen et al. 1994; Walter et al. 1995). These factors may well work in conjunction with remodeling activities to help increase factor binding and perhaps to complete the formation of an open chromatin state by removing core histones from active regions.

Nucleosome positioning

Both repressed and activated promoters frequently contain nucleosomes that are confined to a discrete position or a set of positions (Richard-Foy and Hager 1987; Thomas and Elgin 1988; Wolffe and Brown 1988; Fragoso et al. 1995). Positioning has been proposed to repress expression by placing regulatory sites in an inaccessible place on the nucleosome and has been proposed to enhance expression by juxtaposing activator binding sites (for review, see Lu et al. 1994). The DNA sequences that determine positioning in natural promoters are not well understood, although there is a clear bias toward AT-rich sequences in the minor groove adjacent to the core region and GC-rich sequences facing away from the core. Sequence-specific binding proteins almost certainly can contribute to positioning. Recapitulation of appropriate positioning in vitro will be essential to understanding how nucleosomes might be repositioned by regulatory complexes and to understand whether repressive complexes stabilize positioning.

Linker histones and higher order structures

The nucleosome is the primary mode of packaging in chromatin; binding of linker histones (i.e., histones H1 and H5), formation of the 30-nm fiber, and formation of higher order structures are also essential components of chromatin structure. Histone H1 is not found in yeast, so its role in establishing repressed complexes is not yet clear; specific repressive complexes are not yet characterized in multicellular eukaryotes that contain H1. The effect of H1 on ATP-dependent remodeling in *Drosophila* extracts has been examined (Tsukiyama et al. 1994; Varga-Weisz et al. 1995). H1 decreases, but does not eliminate, the ATP-dependent stimulation of GAGA factor binding and the ATP-dependent stimulation of restriction enzyme access. These data suggest that H1 inhibits function of these remodeling activities, although it is possible that the remodeling activities are fully active and H1 directly interferes with factor access. Addition of histone H5 has been shown to decrease nucleosome mobility and repress RNA polymerase III transcription (Ura et al. 1995), providing a possible mechanism for repression of RNA polymerase II transcription by H1 (Kamakaka et al. 1993). In theory, formation of chromatin structures even more compacted than those formed with nucleosomes and H1 should severely restrict factor access and remodeling activities. This has not been addressed.

Summary and perspectives

Recent studies have provided strong evidence that macromolecular complexes are used in the cell to remodel chromatin structure during activation and to create an inaccessible structure during repression. Although there is not yet any rigorous demonstration that modification of chromatin structure plays a direct, causal role in either activation or repression, there is sufficient smoke to indicate the presence of a blazing inferno nearby. It is clear that complexes that remodel chromatin are tractable in vitro; hopefully this will allow the establishment of systems that provide a direct analysis of the role that remodeling might play in activation. These studies indicate that establishment of functional systems to corroborate the elegant genetic studies on repression might also be tractable. As the mechanistic effects of these complexes are sorted out, it will become important to

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understand how the complexes are regulated. In many of the instances discussed above, the genes whose products make up these complexes were identified in genetic screens for effects on developmental processes. This implies a regulation of the activity of these complexes in response to developmental cues and further implies that the work to fully understand these complexes will occupy a generation of scientists.

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GTF fractions, further fractionation would likely separate the activity and allow direct evaluation of the effect of hyperacetylated nucleosomes on activated transcription in vitro.

(e) Summary for aim 2

The experiments designed to examine the effects of hSWI/SNF (alone and in combination with histone hyperacetylation) on activator binding to nucleosomal DNA rely on established methods and activator preparations that have already been made, can be made by similar protocols, or will be available through collaboration. These experiments should be relatively straightforward and will increase our understanding of how activator binding to DNA can be facilitated and what role the activation domain plays in this process. The experiments designed to examine whether hSWI/SNF and/or histone hyperacetylation can increase binding of GTFs to nucleosomal DNA or stimulate transcription by activators in vitro are more technically demanding, and unlike the experiments to this point, we do not know that we will see an effect. However, the work of Lorch et al (1992) clearly demonstrates that GAL4 activators and highly purified GTFs are not sufficient for transcription of nucleosomal templates; some factor(s) present in crude extracts are also required. In addition, the genetic and biochemical analyses discussed in the background section all suggest that hSWI/SNF and acetylase activities that result in hyperacetylated histones are excellent candidates for factors that facilitate activator function in chromatin. Therefore, it is important for us to investigate whether these factors directly affect transcription factor binding and transcription initiation, and, if so, to characterize the mechanisms responsible for the effects.

Aim 3: Effects of hSWI/SNF components on transcription initiation in vivo

Transfection of cells with the hSWI/SNF components BRG1 or hBRM stimulates transcriptional activation by glucocorticoid receptor (GR), retinoic acid receptor (RAR), and estrogen receptor (ER) (Khavari et al., 1993; Muchardt and Yaniv, 1993; Chiba et al., 1994; Singh et al., 1995). Transfection with genes containing mutations in the ATP binding site inhibited or reduced stimulation by these activators (Khavari et al., 1993; Muchardt and Yaniv, 1993). Thus these mutants act as dominant negatives for gene activation, suggesting that the wild-type BRG1 and hBRM play a role in the process by which these activators stimulate transcription. Other experiments show that the ability of GR and ER to stimulate transcription when introduced into yeast requires yeast SWI and SNF proteins, and that GR can be co-immunoprecipitated from cell extracts with the yeast SWI3 protein (Yoshinaga et al., 1992). However, there are currently no data that identify specific mammalian genes that require hSWI/SNF activity for appropriate transcriptional regulation and/or initiation in vivo. To address whether hSWI/SNF affects the activation of mammalian genes, cell lines that inducibly express dominant negative hBRM and/or BRG1 proteins will be constructed. Much of the available evidence suggests that genes responsive to nuclear hormone receptors are good candidates for regulation by hSWI/SNF, therefore emphasis will be placed on examining genes responsive to these receptors.

(a) Establishment of cell lines that inducibly express dominant negative hBRM and BRG1 proteins

Conditional expression will be achieved using the Tet repressor system established by Gossen and Bujard (Gossen and Bujard, 1992), with the modifications of Shockett et al (Shockett et al., 1995). Briefly, cells will first be stably transformed with a vector that encodes a Tet-VP16 regulatory protein, which is a fusion of the DNA binding domain of the Tet repressor with the activation domain of the herpes simplex virus activator VP16, and a vector encoding a gene for puromycin resistance, for selection purposes. The fusion protein is active in the absence of tetracycline or at low tetracycline concentrations and can therefore stimulate expression from promoters containing a binding site for Tet repressor. Since the Tet repressor is inactivated (unable to bind DNA) at high tetracycline concentrations, expression of promoters containing a Tet repressor binding site is shut off when high concentrations of tetracycline are added to the media. Initially, 12-24 puromycin resistant colonies will be isolated and expanded and will be tested for Tet-VP16 expression by transfection of a luciferase reporter under the control of Tet binding sites. A cell line expressing high levels of luciferase activity in the absence but not the presence of tetracycline will then be transfected with a hygromycin resistance marker and the pTet-Splice vector (Shockett et al., 1995) containing the dominant negative version of hBRM or BRG1 protein and incubated in the presence of puromycin, hygromycin and tetracycline (to prevent expression of the dominant negative protein).

Dominant negative versions of BRG1 and hBRM were obtained from G. Crabtree and M. Yaniv, respectively, and the mutation from each was cloned into Bluescript plasmids containing wild-type BRG1 or hBRM proteins that are epitope tagged with the FLAG epitope (DYKD) at the C terminus (Sif and Kingston, unpublished). The epitope tagged, dominant negative version of each protein was recognized by the anti-FLAG antibody M2 (commercially available from Kodak) following in vitro transcription/translation and was then

cloned into the pTet-Splice vector. Initially, 12-24 colonies resistant to puromycin and hygromycin will be expanded and tested for expression of the dominant negative protein in the absence but not the presence of tetracycline by western blot with the M2 antibody in order to distinguish the introduced proteins from endogenous protein. To confirm expression, M2 antibody bound to beads (Kodak) will be used to immunoprecipitate the dominant negative proteins. The immunoprecipitated material will then be used for a western blot and probed with antibodies that have been generated to BRG1 and hBRM (unpublished). Cells expressing high levels of the dominant negative protein will be selected for further study. To characterize dominant negative protein production, protein levels will be monitored by M2 in western blots as a function of tetracycline concentration and time. Lines that express each dominant negative individually will be made. In addition, I will make a line that expresses both mutant BRG1 and mutant hBRM. To do this, cells expressing Tet-VP16 will be transfected with the hygromycin marker and both of the pTet-Splice plasmids that contain the dominant negative proteins. Selection will be as described above, except that following immunoprecipitation of puromycin and hygromycin resistant cell extract with the M2 antibody, the immunoprecipitates will be probed for expression of both BRG1 and hBRM using the anti-BRG1 and anti-hBRM antibodies.

Conditional expression of the mutant proteins has been chosen for these experiments for several reasons. First, it allows direct comparison of gene expression in the same cell line before and after induction of dominant negative BRG1/hBRM. Second, the Tet repressor system will allow a range of expression levels to be achieved simply by altering the tetracycline concentration. Bunker and Kingston have demonstrated that when GAL4-VP16 is tet regulated, a linear increase in GAL4-VP16 protein levels can be observed as tetracycline concentration is lowered (Bunker and Kingston, 1996). Thus, in theory, any results that are obtained can be compared at different levels of effector protein (the dominant negative). Thirdly, it alleviates the potential problem of the induced protein affecting cell division and growth. This is particularly relevant for BRG1 and hBRM. Both proteins have been shown to bind to the retinoblastoma (Rb) oncoprotein and can induce cell cycle arrest in certain cell lines in an Rb dependent manner (Strober et al., 1996; Dunaief et al., 1994).

(b) Effects of dominant negative proteins on induction of MMTV LTR by GR

If expression of the dominant negative hBRM or BRG1 proteins affects gene expression in vivo, ideally, one would like to examine any effects seen in vivo at the level of promoter structure on the affected gene(s). One of the best characterized eukaryotic promoters is the mouse mammary tumor virus long terminal repeat (MMTV LTR). A tremendous amount of work has been reported on activation of MMTV LTR by glucocorticoids, on the factors that bind to the LTR, on the defined chromatin structure the LTR assumes upon insertion into the genome or into stably replicating DNA, and the structural changes that occur in chromatin structure upon activation of transcription. Given that much of the evidence that examines hSWI/SNF regulation in eukaryotic cells strongly suggests that nuclear hormone receptors such as GR may be affected by hSWI/SNF, I propose to examine the effects of expression of dominant negative BRG1 and/or hBRM proteins on MMTV LTR activation by GR.

When stably introduced into cells, the MMTV LTR becomes organized into an array of six positioned nucleosomes (Richard-Foy and Hager, 1987). Induction of expression is generally dependent upon addition of steroid hormones, and, in the case of glucocorticoid-mediated stimulation, is accompanied by a disruption or alteration of the nucleosome encompassing the GR binding sites and binding of the transcription factor NF-1 and the basal transcription machinery to the promoter (Richard-Foy and Hager, 1987; Zaret and Yamamoto, 1984; Cordingley et al., 1987; Archer et al., 1992). Though GR can bind to reconstituted nucleosomes in vitro, NF-1 is apparently excluded (Perlmann and Wrange, 1988; Pina et al., 1990; Archer et al., 1991). Thus, the MMTV promoter possesses a defined chromatin structure that prevents the binding of a number of transcription factors required for expression and transcriptional stimulation by GR results in an alteration of nucleosome structure to facilitate loading of NF-1 and the GTFs to the promoter.

3

Experiments Initial experiments will be done using a human mammary carcinoma derived cell line called T47D(A1-2) (Archer et al., 1994). This cell line, as well as any technical support needed, will be provided by Dr. Trevor Archer (Univ. Western Ontario- see letter- Appendix X). Dr. Archer characterized the properties of the A1-2 line, has a great deal of experience examining steroid hormone activation of the MMTV LTR, and has refined many of the assays used to examine MMTV chromatin structure. A1-2 cells have been engineered to constitutively express comparable levels of both glucocorticoid and progesterone receptors, and they also possess approximately 10 copies of a stably integrated MMTV luciferase reporter gene. Addition of dexamethasone to these cells greatly induces GR mediated transcription, increases restriction enzyme accessibility to nucleosomal MMTV sequences, and facilitates loading of NF-1 onto the promoter. In contrast, addition of progesterone or the synthetic progestin R5020 induces a small but measurable amount of PR

mediated transcription. The lack of significant PR mediated transcription correlated with a failure to increase restriction enzyme accessibility and failure to promote NF-1 binding to the promoter (Archer et al., 1994).

The Tet repressor system and dominant negative BRG1 and/or hBRM vectors will be introduced into these cells as described above. Cells will be maintained in G418 in addition to the drugs indicated above, as the MMTV construct that was integrated was cotransfected with a vector conferring G418 resistance. Duplicate cultures will be incubated in the presence of tetracycline, thereby preventing expression of the dominant negative or in either the absence of or at low tetracycline concentrations in order to induce production of the dominant negatives. At a time determined to be sufficient for expression of the dominant negative protein, cells will be incubated in the presence or absence of dexamethasone. Expression from the MMTV LTR will be monitored by measuring luciferase activity and by analyzing mRNA levels by S1 or primer extension analysis. mRNA levels will be normalized to expression levels of a gene unaffected by hormone induction such as actin. If effects of the dominant negatives on MMTV expression are observed, the promoter structure will be analyzed using published assays. To examine restriction enzyme accessibility, isolated nuclei will be exposed to Sst I or Afl II, both of which cleave near receptor recognition sequences in a region of the promoter that is incorporated into a nucleosome. In addition, there is a second Sst I site that is located in a region of the promoter that lies between two nucleosomes. This site has been shown to be accessible to Sst I and is not affected by hormone addition, so cleavage at this site can serve as a control for digestion efficiency (Archer et al., 1994). Following digestion, genomic DNA will be isolated and amplified using Taq polymerase and a ^{32}P end-labelled oligonucleotide specific for the MMTV LTR, as described (Archer et al., 1991), and resolved on polyacrylamide denaturing gels. Finally, the extent of NF-1 loading onto the promoter will be examined using an in vivo exonuclease III footprinting assay as described (Cordingley et al., 1987; Mymryk and Archer, 1994). For this assay, nuclei will be isolated after cells are appropriately treated with tetracycline and/or dexamethasone and exposed to exonuclease III and Bam H1, which cleaves upstream of the MMTV sequences and thereby provides an entry point for the exo III enzyme. After isolation of the genomic DNA and Taq amplification, bands will be resolved on polyacrylamide denaturing gels. The appearance of a dexamethasone induced band(s) adjacent to the NF-1 binding site signifies NF-1 binding.

In vivo footprinting assays using DMS or DNAse I to detect protection of residues by NF-1 will also be attempted, however, this technique has proven difficult with rodent lines containing MMTV sequences in the genome or in stably replicating episomes, as apparently not all copies of the promoter are active (T. Archer, personal communication). The presence of inactive (and presumably unoccupied) promoters would not affect the exo III assay, as occupation of the binding site by NF-1 is observed as the appearance of bands on a gel. In contrast, in a DMS or DNAse I protection assay, sequences in unoccupied promoters would be cleaved and DNA species resulting from these cleavages would interfere with visualizing protection of these sequences on active promoters. Whether some of the MMTV copies are inactive in T47(A1-2) cells is not known.

The experiments will also be repeated changing the order of the inductions to determine whether BRG1/hBRM are involved in the maintenance of the activated state. Cells will first be exposed to dexamethasone and subsequently have the tetracycline washed away. This will allow establishment of the GR activated state prior to induction of the dominant negative protein. If no dominant negative mediated inhibition of transcription is observed, it will suggest that the effect of the dominant negative was at the level of promoter structure, and will suggest that once the promoter structure is made accessible for the transcription factors, there is no further requirement for the hSWI/SNF proteins. In contrast, if dominant negative mediated inhibition of transcription is observed, it will suggest that there is an ongoing requirement for hSWI/SNF proteins. If the dominant negative were affecting the ability of the promoter to stay in the altered or open confirmation that is associated with induced transcription, this should be reflected by a decrease in restriction enzyme accessibility and NF-1 loading on the promoter.

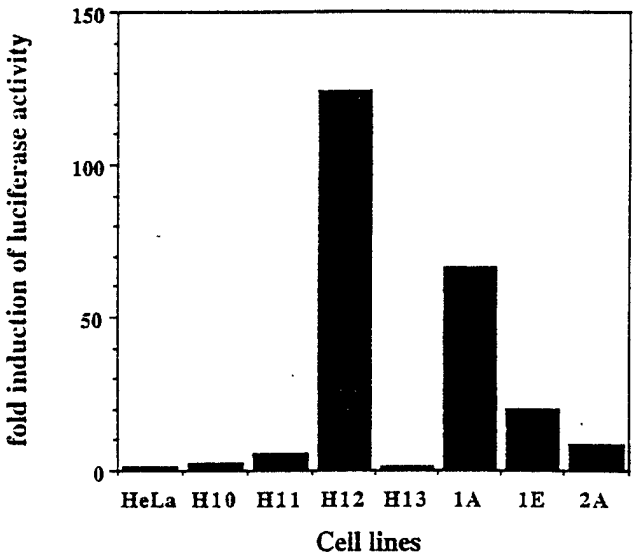
(c) Identification of cellular promoters affected by dominant negative proteins

These cells will also be used to identify cellular genes that are affected by mutant hSWI/SNF proteins. Since in yeast, SWI/SNF affects genes that are rapidly inducible in response to environmental signals, candidate promoters that are rapidly induced by various agents (e.g.- serum stimulation of c-fos, estrogen stimulation of c-myc) will be assayed. If candidate promoters are not identified by the means outlined above, differential display techniques could be used to identify target genes. This approach may identify genes whose promoters are not cloned or characterized, making further analysis more difficult, however, it will provide an unbiased screen to identify genes that are affected by hSWI/SNF proteins in vivo.

(d) Isolation of mutant hSWI/SNF complex

Mutant hSWI/SNF complex will be purified for use in the in vitro assays in aims 1-3. Since even small scale purification requires relatively large quantities of cells, an analogous cell line(s) expressing dominant negative hBRM and/or BRG1 will be created from HeLa spinner cells. Since these inducibly expressed proteins are epitope tagged, purification of the mutant complex will be facilitated and possibly simplified. I have recently started construction of the HeLa lines. The tet-VP16 regulator was transfected with a drug resistance marker and drug resistant colonies were picked and expanded. These lines were assayed for the ability to stimulate expression from a transfected luciferase reporter containing tet repressor binding sites. Fig. 11 shows induction of luciferase expression in several of the lines. Incorporation of dominant negative BRG1 and hBRM into H12 cells is currently in progress.

Fig. 11



(e) Summary of aim 3

All of the genetic and biochemical data suggest that hSWI/SNF functions to alter chromatin structure and facilitate transcription in vivo, but direct evidence of these roles on a specific promoter in higher eukaryotes has not been demonstrated. The existing data strongly suggest that hSWI/SNF is involved in activation of transcription by steroid hormone receptors; the tremendous advantage of examining MMTV expression in the manner proposed is that the in vivo chromatin structure of the MMTV LTR has been already been defined in detail. Thus analysis of both expression and changes in in vivo chromatin structure in response to GR and altered hSWI/SNF proteins can be performed using established techniques and assays. There are very few promoters in higher eukaryotes that have been characterized in vivo in any detail, so while it is possible to look at effects of expression of the dominant negative hBRM and/or BRG1 proteins on any number of genes whose expression can be stimulated, concomitant in vivo study of changes in promoter structure would be much more difficult.

Human Subjects

None.

Vertebrate Animals

One method that may be used to assemble chromatin in vitro utilizes chromatin assembly proteins purified from frog eggs. Vertebrate eggs have high concentrations of assembly proteins, as the eggs will undergo rapid cell division upon fertilization. Frog eggs are used because frogs can be induced to lay eggs by hormone injection, they lay large quantities of relatively large eggs, and they are easy to care for. A colony of approximately 12 adult female frogs will be maintained in the UMass Medical Center animal facility. For a typical preparation, 3-6 frogs will be placed in isolation tank and induced to lay eggs. Following egg laying, the frogs are returned to the main tank. The frogs are not harmed or sacrificed, and their eggs will regenerate in about three months.